EXPERIMENTAL CELL RESEARCH

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THE MICROSTRUCTURE OF RAM SPERMATOZOA

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A. INTRODUCTION

The structure, origin and development of gametes of animals are of fundamental biological importance and interest. The animal egg usually contains large quantities of reserve food material in the form of yolk, and is consequently in the nature of a giant cell comparatively unsuitable as such for physical studies of the type dealt with in this paper. The spermatozoon, on the other hand, is a much smaller cell with marked differentiation and structure. While the sperms of different animals exhibit great diversity in size, form, and detailed structure, there is, as Wilson has pointed out (19), a fundamental common plan of organisation. Broadly speaking, the animal sperm is a motile flagellate cell consisting of a comparatively small dense head, and a long flagellum or tail. Between these two distinct portions of the cell there is often to be seen a middle or connecting piece.

A reading of the classical studies of spermatozoa, such as those of Retzius (14), indicates that while the light microscope has in many instances enabled us to lay down the broad structural plan, there are doubtless many features which can only be elucidated by investigation at much higher resolution. For this purpose the electron microscope is one of the most valuable contributions of the physicist to the study of biology. Already Bretschneider (3) and others (12, 8, 16) have made preliminary studies of certain animal spermatozoa by this means.

In the use of a new instrument, such as the electron microscope, on biological material the danger of artifact production is increased by the necessity of examining the specimens in vacuo. At the same time, the order of size of the sperm is such that a number of features and dimensions can always be checked by light microscope measurements on living material (see section

F). Moreover, the recent and increasing territory common to X-ray diffraction and electron microscopy has made it quite clear that one of the most profitable uses of the electron microscope lies in the investigation of those structures which show features of molecular orientation, since such features can frequently be checked by other means. The crystallinity of certain viruses (10), the identity of the protein molecules in such structures as edestin, and the globular-fibrous transition in proteins (13) are all cases in point. On these grounds, spermatozoa would appear to be among the more suitable items of biological material for detailed electron microscope studies. Finally, the active progress now being made in the field of the metabolism of semen (9) gives the hope, however distant, that it may be possible by suitable design of experiment to link the structural features of sperm with biochemical nature and function, thus extending knowledge beyond the immediate range of cytology.

Our choice of ram spermatozoa as the material for these investigations is partly linked with these considerations. As Mann has pointed out, ram semen is distinguished by a very high concentration of spermatozoa, $2–5 \times 10^6$ per microlitre of semen, although the volume of a single ejaculate (0.7–2 ml) is small. In this respect ram semen is a much more suitable material for metabolic studies than either bull or human semen. From the purely morphological point of view ram semen is found to be remarkably constant, in that abnormalities of structure are comparatively rarely encountered in the sperm. While typical of mammalian sperm, the head of the ram gamete is not as opaque to the electron beam as that of many other species[1], and detailed examination has thus been possible. Preparation of sperm for examination in the electron microscope was assisted by the fact of high concentration already mentioned. In the case of human sperm the presence of large amounts of accessory secretions makes purification more difficult.

The present paper is an account of our experiments and conclusions concerning the detailed structure of ram spermatozoa as examined chiefly, but not entirely, in the Siemens electron microscope; it will be seen from this first account that the complexity of structure is much greater than has often been supposed. For this reason, and because we have not yet had the opportunity to study spermatogenesis or to develop the thin-section techniques so obviously required, our conclusions must be regarded as tentative, particularly in relation to fine detail. A preliminary account of this work was pre-

[1] Preliminary experiments on rat, herring, plaice, and sea urchin spermatozoa reveal sperm heads extremely opaque to electron beams of the energy of 10^5 electron-volts or less; the heads of human sperm are less opaque but not so transparent as those of ram and bull.

sented at the International Electron Microscope Conference, Delft, 1949. While we believe that these experiments carry our knowledge of the microstructure of the sperm a good deal further, it is clear that much work remains to be done before anything like a complete picture can be given.

B. PREVIOUS WORK WITH THE LIGHT MICROSCOPE

It is not possible here to give a detailed account of previous work in this field, but a brief summary must be attempted. The work of Retzius has already been referred to. Ballowitz (1), Bowen (2), Gatenby (6), Collery (5), Gresson (7), and others have all made detailed studies of spermatozoa and spermatogenesis with the light microscope on a wide range of species. Gresson has given attention to ram spermatozoa and spermatogenesis in his 1945 paper. We are here concerned with mammalian spermatozoa and reference to Fig. 1 gives some broad indication of the structure of the mature sperm as determined by standard cytological studies in the light microscope. The relative dimensions of Fig. 1 refer only to ram sperm. The description which follows is derived chiefly from the works of Wilson and Gresson. The head varies in size and form in different animals and the nucleus of the gamete cell constitutes the main part of the head.

Ultraviolet studies (4) have shown one of the chief constituents of the head to be nucleic acid. The acrosome assumes different forms in different species; in the guinea pig (see the publications of Gatenby and Gresson) it is a blunt cap-like structure divided into an inner and outer zone; in rodents it is curved and hooklike. The posterior part of the nucleus is covered by the post-nuclear cap and the whole head, indeed the whole cell except for a possible bare tail-end, is said to be enclosed in a thin protoplasmic sheath.

The neck, the most fragile part of the cell[1], connects the head and middle-piece and is bounded by a continuation of the protoplasmic sheath of the head. The axial fibre begins at the neck and is continued through the middle-piece and flagellum. A centriole serves as a basal granule for the axial fibre. Earlier work also gives evidence that the axial fibre is built up of fine fibrils, and that it is a contractile structure responsible for the movement of the tail. Golgi material is said to be present in the neck.

That the middle-piece is usually thicker than the flagellum is due to the

[1] Many types of mechanical treatment, such as those referred to on p. 6 all break the sperm in this region. It is progressively more difficult to disintegrate the sperm tail and head.

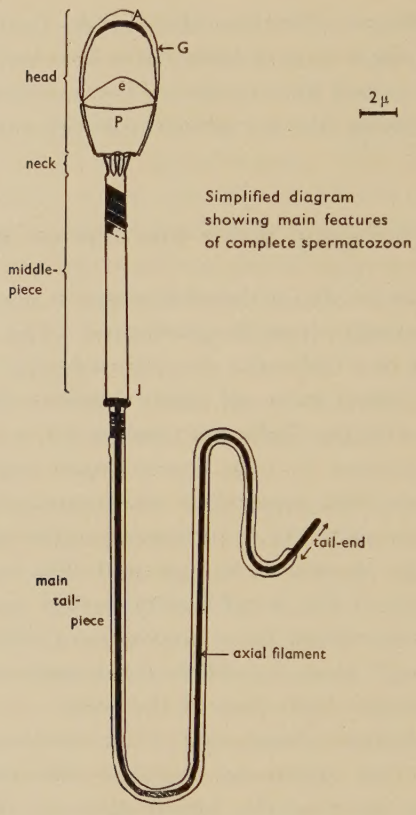


Fig. 1. Simplified diagram of the main features of a complete ram spermatozoon.

presence of a sheath which originates in the mitochondria of the spermatid; this sheath is known as the mitochondrial sheath and often appears to have a helical structure. The posterior limit of the middle-piece is determined by a ring centriole (Jensen's ring).

The flagellum is made up of two portions, the main-piece and the end-piece. The main-piece is the longest part of the flagellum and is composed of the axial fibre surrounded by the protoplasmic sheath. The end-piece consists of the terminal part of the axial fibre and is not enclosed by the sheath.

The work of Schmidt (15) and others has shown conclusively that sperm-heads contain strongly doubly-refracting oriented nucleic acid material. Polarized light photographs taken recently in this laboratory (Plate VIII and section F) show quite clearly the presence of a helical component in the structure of the middle-piece of live ram spermatozoa.

C. PREPARATIVE DETAILS

1. *General*

It is simplest to refer here to the final stages of the preparation and mechanical treatment.

The usual procedure consisted in placing a drop of the treated suspension of sperm on the (formvar) film-covered grid by means of a small platinum loop. The tendency for a drying drop to cause excessive deposition of material at and near the centre can be avoided by the use of a large drop, which is subsequently drained by touching the edge of the grid with filter paper.

The avoidance of outside contamination and strict observance of great cleanliness in the preparation of material are of the utmost importance if misleading complications are to be avoided. For this reason repeated washing of the suspension is preferred to washing of the preparation when finally on the grid; the latter method is not only less reliable, but also tends to cause local strain and creasing of the drying film.

2. *Treatment of Semen*

Ram semen was obtained from Dr. A. Walton of the Department of Agriculture, Cambridge, where it was collected by means of the artificial vagina technique and subsequently cooled to approximately 5° C. On arrival in the laboratory in London some 6–8 hours later the material was generally allowed to return to room temperature before being treated.

The sperm concentration of the semen, as already mentioned, is $2–5 \times 10^6$ per mm^3, with a range of pH of 6.8 ± 0.5. The nature of the diluent depends on whether it is used before or after fixation. If the former, Tyrode solution or a phosphate buffer was employed; if the latter, and the fixative was in solution, the application of excess fixative provided the diluent. In other cases, such as the use of vapour fixative, subsequent dilution was effected by a phosphate buffer.

The purification of the material is chiefly necessary in order to eliminate mucus and other matter that tends to become attached to the sperm, thus complicating interpretation by obscuring the structure. The purification consists in removal of the semen plasma by washing with Ringer solution; routine washing was done by repeated centrifugation for two minutes at 1,500 rpm. Throughout the experiments wet and dry samples were examined and checked under the light microscope (oil immersion).

3. *Fixation*

The following fixatives were used: (1) formalin, (2) osmium tetroxide vapour, (3) phosphotungstic acid, (4) air-drying.

Formalin. A 5 per cent solution of formaldehyde in water or Tyrode was used.

Osmium tetroxide vapour. The osmic acid (2 per cent) was contained in a small dish, and a coverslip with small drops of semen on it was inverted over the dish for a known time (30 sec.–2 min.). The fixed semen was then washed off with distilled water or phosphate buffer.

Phosphotungstic acid was also used as a fixative in the form of a 0.2 per cent solution adjusted to neutrality by means of sodium carbonate. This solution was applied as a large drop to the electron microscope grid which held the specimen, and the rate of evaporation was controlled by enclosure under an inverted Petri dish. Removal of excess phosphotungstic acid was achieved by floating the grid face downwards on a clean water surface.

Bulk fixation was sometimes adopted, and in such cases the whole sperm suspension was made up to give a 0.2 per cent solution on the addition of phosphotungstic acid. Washing was carried out by centrifuging at 2,500 r.p.m. for 2–5 minutes at a time.

Air-drying. Drops of washed sperm suspensions were placed on grids and allowed to dry either in a desiccator over calcium chloride or in a vacuum desiccator; in general there was no difference between these two techniques.

4. *Stains*

Osmium tetroxide and phosphotungstic acid were used as stains on formalin fixed material.

5. *Enzyme Digestion*

Trypsin digestion of unfixed and alcohol-fixed sperm was carried out at 18° C, and samples removed every 24 hours. (One drop of semen was diluted with 5 cc of phosphate buffer solution and 5 mg of trypsin as supplied by Fairchild Bros. and Foster.)

6. *Mechanical Treatment*

Two different methods have been used in order to break down the sperm structure by mechanical means:

(a) *Mechanical shaker in conjunction with glass beads.* This method is due

to Dr. T. Mann, and is a means of separating tails from heads, the process taking anything from 36 to 48 hours. The method, coupled with subsequent dilution and centrifugation, has provided a means of examining heads from which some material, chiefly ribosenucleic acid and phospholipids, has been removed. We are indebted to Dr. Mann for a number of preparations of this type.

(b) *Ultrasonics* provides a readily controlled means of loosening or breaking up small structural units such as sperm. Zittle and O'Dell (20) have already made use of the method in biochemical studies of bull sperm and Schmitt (16) has reported a sub-divided human sperm tail and an isolated helix got by this means. The generation of ultrasonic waves in a liquid under conditions allowing cavitation means that large stresses can be applied to cells in suspension. In the present experiments apparatus designed by Selman and Wilkins (17) has been used. The diluted semen was placed in a small glass tube provided with a cellophane base. The tube was contained in an outer vessel filled with isotonic solution to avoid osmotic effects. The outer vessel was separated from the oil surrounding the transducer by a thin membrane to minimise absorption. Water cooling of the inner tube ensured that the temperature was kept at 15° C. It was usual to dilute the semen twenty times for ultrasonic treatment.

7. *The Electron Microscope and the Shadowing Technique*

The instrument used was a Siemens model with a maximal operating voltage of about 90 kV; most of the work described in this paper was carried out at 70 to 80 kV.

The metal shadowing of specimens is now well-known and was carried out in accordance with the technique introduced by Williams and Wyckoff (18). Palladium-gold alloy was generally used in preference to gold which was found to aggregate rather badly. Palladium alone was sometimes used but was not quite as satisfactory. The angle of shadowing ranged from 15° to 45°; most of the work was done at a shadowing angle of 25°.

It is essential, particularly when dealing with such very fine structural detail, that the confusion in interpretation due to metal aggregation should be kept at a minimum; for this reason comparatively low magnifications in the neighbourhood of 10.000–12.000 have chiefly been used. Higher magnifications involve higher electron beam intensities and a greater tendency to aggregation. A deflector-focusing device of the type introduced by the Philips laboratories, Eindhoven, makes it possible to keep the intensity low.

D. TERMINOLOGY

The classical terminology used to describe the various visibly distinct parts of the sperm does not suffice in the present investigation in which many new features have been observed. At this interim stage it seemed in general undesirable to give names to the new features and refuge has been taken in lettering, in many cases with numbered suffixes. A complete list has been given in the appendix on terminology. An exception has been made in the case of what has previously been called the *axial filament.* In view of the much greater detailed information now available we consider it more appropriate to use the terms *fibre, fibril,* and *filament* to describe the successive stages of sub-division. The use of these terms should be clear from the text below.

E. RESULTS

In this section we shall describe the main results of the paper with the help of electron micrograph reproductions and detailed drawings. It will be understood that while the conclusions are based on detailed examination and measurement of almost a thousand plates, only a few reproductions are possible; and that, even with the highest class of reproduction, the full details of the original plates cannot reach the printed page. In an investigation of this nature one is fortunate if a given plate shows more than a few features of importance. The final integrated structure derived from a study of all plates must therefore contain much more information than can be seen by examination of a few reproductions. This is inherent in the method of publication; the greatest care has been taken to include only items of information and structure which we consider to be well-established. Less established features will be indicated in the description. It is appropriate to discuss first the head, then the tail-end and main-piece; and finally the middle-piece and neck.

E. 1. The Sperm-Head. Initially one would have expected to gain few new facts about the sperm-head structure without the aid of special sectioning techniques. Our choice of sheep sperm is fortunate in that the head appears to be less absorbent for electrons than that of human, and even bull sperm. As a consequence a good deal of detail may be seen in the electron micrographs. As a first example reference should be made to Plate 1 a. The shape and size of the head as shown in this photograph (length 8.2 μ and

width 4.25 μ) are approximately the same as those observed from light microscope pictures of living ram sperm.

The specimen was "shadowed" and it should be stressed that this technique has special value here in revealing fine surface structure, although this does in some ways obscure underlying features.

E. 1 a. The Galea Capitis is a thin sheath-like covering extending over the anterior portion of the head. Its termination coincides with the anterior boundary of the equatorial segment. Drying effects have shown the galea capitis to be closely attached to the sides of the head, and detached from the tip. Osmic acid and phosphotungstic acid fixation have been found very effective in preserving the galea capitis and in showing up the great complexity of its structure; formalin is much less satisfactory.

The galea capitis has three components in its structure:

(a) a transparent membrane
(b) a fibrous component and
(c) a globular component.

The transparent membrane is revealed quite well in shadowing experiments and is ~100 mμ in thickness. Three types of fibre have been observed in the galea capitis and it is uncertain whether they are superposed, interwoven, or underlying the membrane. The first type of fibre can be seen in Plate 1 a and b; it is coarse, probably complex, and widely and irregularly distributed. The thickness of these fibres is 40–80 mμ. The second type of fibre is rather finer, 20–30 mμ in diameter, more widely distributed and superposed on the first type. The third class of fibre, more strictly filaments, is very fine indeed (cf. Plate 1 b), ~5–10 mμ, and the system forms a very close network over the whole galea which is very difficult to see, but undoubtedly present (cf. Plate 1 d).

Various sizes of globule have been observed in the anterior part of the head structure, and it is necessary to guard against artifacts caused by (i) the precipitation of material on the sperm structure and (ii) the aggregation of metal under electron bombardment and the consequent simulation of globules. The first type of artifact can be dealt with and assessed by the use of various methods of preparation; the second by the use of the minimal intensity of electron beam compatible with accurate work. A check on aggregation effects can be obtained by taking photographs while exposing a test specimen to various intensities and comparing the sizes of globule obtained. Further comparative information can often be derived from a study of the background (cf. G. Discussion).

Complex questions of the position and size of the globules immediately arise. The considerable range in size, i.e.

100–300 mμ..... (large)
60– 80 mμ..... (medium)
20– 30 mμ..... (small)
10 mμ and less ... (very small)

suggests that there are distinctly different types of globules. The very large globules have been flattened to the extent of presenting an almost ring-like appearance and may lie underneath the galea capitis itself. Medium sized globules appear as disks, and there are indications that the disks are linked with fibrils of type 2. These globules are visible on Plate 1 c. Smaller globules retain their spherical shape and are more often found in the equatorial segment than elsewhere (cf. II c and I d). They may therefore be quite independent of the galea capitis and, instead, form part of an intermediate structure between the acrosome and galea capitis. No complete intermediate structure has so far been observed. A fourth type of globule is smaller and of considerable irregularity in both size and position.

E. 1 b. The Acrosome consists of two parts A_1 and A_2 (Fig. 2); of these A_2 is the larger and fits closely over the head with its posterior boundary roughly coincident with the posterior boundary of the equatorial segment. We refer to A_2 as the acrosome. A_1 is an acrosome cap covering the tip of the head, and is clearly visible in Plate II a where it is partly detached. (A cap has also been observed in optical studies: see section F.) A_1 cannot usually be seen in electron micrographs of specimens where the galea capitis is still attached to the head. Occasionally not only the galea capitis, but the acrosome cap itself is missing, together with the underlying portion of the acrosome (see Plate II b). The nature of the acrosome surface structure is not definitely known but it has a distinctly granular appearance in a number of photographs.

A difficult problem to decide is the origin of certain rather coarse fibrils of diameter 20–100 mμ found overlying the acrosome. These fibrils persist when the galea capitis is missing (Plates III a and I c), and in any case extend over the equatorial segment, and thus beyond the posterior limit of the galea capitis (Plate II c). An open irregular network lying between the galea capitis and the acrosome is thus suggested but by no means proved; this structure is not indicated in Fig. 2.

E. 1 c. The Equatorial Segment has a smooth surface, presumably due to an additional superficial layer. This can be inferred from the fact that

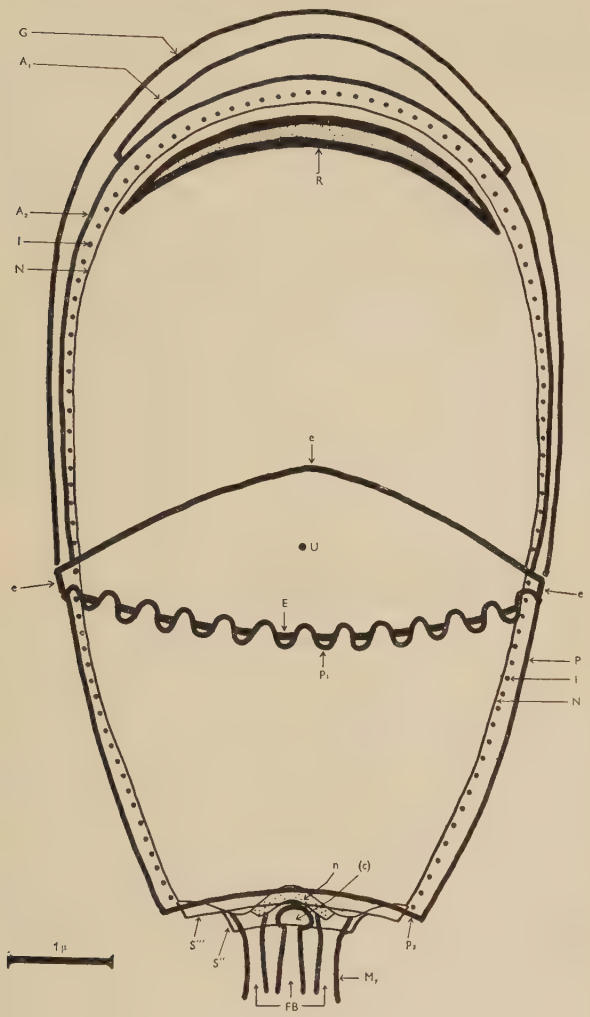


Fig. 2. Diagram of the sperm-head structure.

at each side of the head this segment juts out beyond the head boundaries (see Fig. 2). The anterior boundary of the segment is often very clearly defined (as in Plate I a) and subtends an angle of approximately 120°. The posterior boundary is very clearly defined by a thickening, the nuclear ring, which is 20–30 mμ thick. This ring lies underneath the serrated edge of the post-nuclear cap, the serrations projecting towards the acrosome as shown in Figs. 2 and 3. About half of each serration (total depth 150–200 mμ)

extends beyond the nuclear ring on to the equatorial segment. There are also indications that the nuclear ring is not strictly continuous, and is sometimes seen to give rise to fibrils which continue underneath the post-nuclear cap.

The smooth surface of the equatorial segment is overlaid with fibrils and globules, somewhat similar to those seen on the *galea capitis*. These fibrils are not of the simplest linear character and there are indications that they incorporate globular units in their construction. Careful further observation may disclose early stages in this elementary organisation, which is somewhat similar to that observed by Rudall (13) in the lower layers of the earthworm cuticle.

E. 1 d. Post-Nuclear Cap. The serrated upper edge of the cap has already been mentioned and dimensions have been given in Fig. 3. It is only necessary

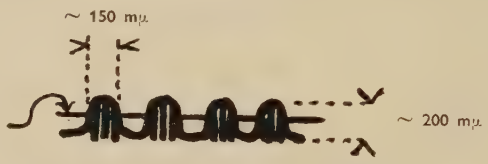


Fig. 3. Enlarged view of a portion of the nuclear ring and the fibrous serrated edge of the post-nuclear cap.

here to emphasize that this serration is quite distinct from the nuclear ring. Previous workers have presumably referred to a combination of both features when using this term. The surface of the cap visible in the electron micrographs consists of a very coarse network, the individual fibres of which are ~ 60–80 mμ in diameter. Finer fibrils are apparent at the edges. The serrations of the anterior edge or ring are themselves fibrous; often three fibrils can be distinguished as in Plate II c. This suggests that the coarse reticulum is probably built up of ~ 30 mμ fibre-units. Each serration is terminated by a rounded portion; occasionally fibres can be seen extending upwards from there into the area of the equatorial segment. Very fine fibrils ~ 10 mμ or less can be distinguished at the edge of the post-nuclear cap. They appear to form a network immediately beneath the reticulum, but may well be an integral part of the post-nuclear cap.

What has sometimes been called the neck-band or basal ring is the posterior termination of the post-nuclear cap and has a width of 25–30 mμ (Plate I a and III b). A ring of such small dimensions could only be distinguished in the electron microscope; it must not be confused with the dense lower part of the head characteristic of ultraviolet photographs (Plate VIII

d), and which appears as a fairly well-defined opaque portion in unshadowed electron micrographs.

E. 1 e. The Intermediate Membrane. The nature of the intermediate membrane, I, situated between the acrosome and nuclear surface membrane N (Fig. 2), is uncertain. Again, there is evidence of both fibrils and globules, but the general effect at present is one of indeterminate structure. Nevertheless, there is no doubt as to its existence.

E. 1 f. The Innermost Surface Membrane, presumably the nuclear membrane itself (marked N on Fig. 2), is smooth as revealed by enzyme digestion. As shown in Plate II d, it is penetrated by the various rodlike structures which are sometimes seen to protrude from the inside of the head.

E. 1 g. By *Characteristic Structures* of the head are meant certain features which have been frequently observed on different occasions and under various conditions. Those features considered to be genuine are mentioned here. Foremost is an arc-like thickening, marked R on Fig. 2, and visible on Plate III a. Further structures are shown on Plates III b, II d, and III c. While no attempt at interpretation is made at present, the symmetry about the major axis is interesting. The structures are originally all well within N, and occur in quite different preparations. There is some indication that the coarser types of structure are visible in phase-contrast photographs.

E. 1 h. The Effect of Ultrasonics on the Head Structure. One of the most characteristic effects of ultrasonics on the head of ram sperm is that shown in Plate II a at the point corresponding to U on Fig. 2. A hole is produced at this point and it is found that the position ratio Un/UA_1 (Fig. 2) is constant and equal to 0.39. The hole is usually produced by quite moderate treatment. More prolonged treatment results in a series of gashes and small holes along the major axis. We interpret the small hole and subsequent rupture under such treatment as evidence of mechanical weakness in this region. Whether the hole signifies a fundamental chemical difference, or structural abnormality is not known. It is possible, of course, that the wall of the sperm head in this region has a special metabolic exchange function with the semen plasma, but there is at present no evidence known to us for this interpretation.

E. 2. Tail-End and Main-Piece. After the sperm-head the greatest complexity of structure is shown in the region of middle-piece and neck. This complex structure is formed around an axial fibre which is the most straightforward starting point for discussion. It is therefore convenient and helpful to begin with the tail-end: as we proceed along the flagellum the complexity increases.

Fig. 4 a.

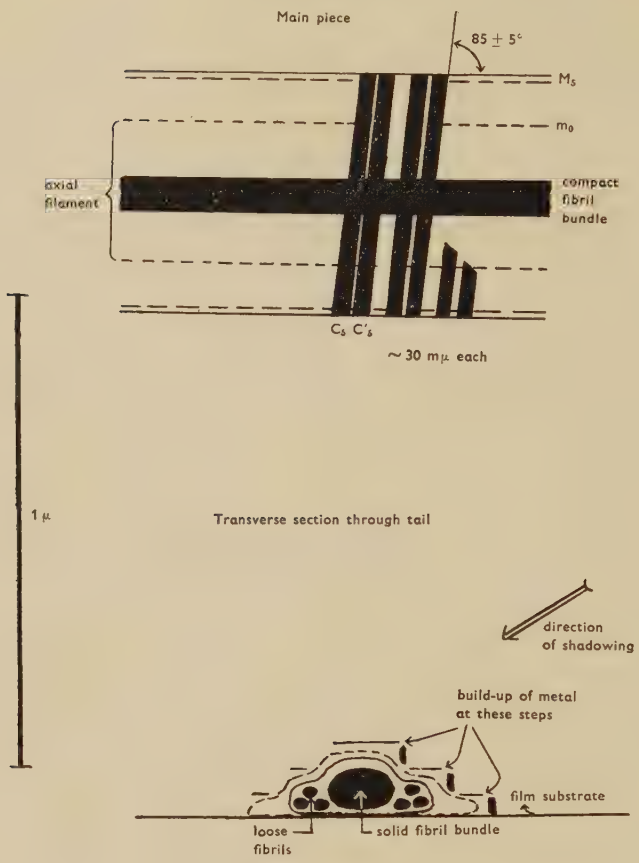


Fig. 4 b.

Fig. 4. Structure of the tail (main-piece and end).

E. 2 a. Tail-End. The axial fibre which runs down the entire length of the tail is encased in a membrane m_o, Fig. 4 c, and finally emerges to form the tail-end, a short portion of about 3 μ in length, and 150–200 mμ wide. The the tail-end is distinguished from the main-piece by a fairly sharp narrowing from ~ 590 mμ to the figure already mentioned. Constituent fibrils of the axial-fibre can be seen to consist of sub-units or filaments of approximately 5–10 mμ in diameter. A pair of these sub-units is enclosed by a coil of thickness 10–15 mμ. This coil makes an angle of 75–90° with the long axis of the sub-unit and is probably two-ply. Such coils can be seen in Plate

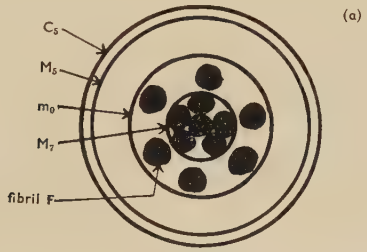


Fig. 4 c.

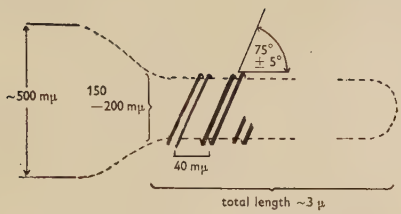


Fig. 4 d.

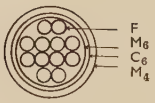


Fig. 4 e.

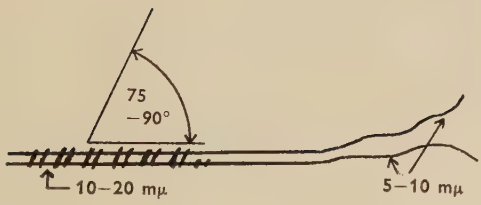


Fig. 4 f.

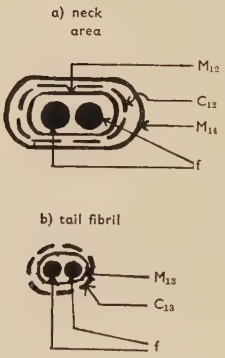


Fig. 4 g.

III d at the points marked by arrows. Individual sub-units of the axial fibrils can be seen in Plate IV a at "breaks" shown by two different tails. The original plates show some indication of structure in these sub-units. The tail-end terminates in a rounded tip indicating only slightly uneven lengths of the closely packed fibrils. The tail-end itself is surrounded by a coil of low contrast C_6 (Plate IV c) 10–20 mμ thick, which envelopes membrane M_6. It is worth noting that a membrane, evidently M_4, representing the lipoid capsule, surrounds the tail-end. It is nearly always missing. M_4 is therefore a continuous covering for the *whole* sperm.

E. 2 b. The Main-Piece is the main and longest part of the flagellum, and is 40–45 μ long and from 500–530 mμ wide. Its chief features can be seen from Fig. 4, a and b. A compact fibril bundle, enclosed by membrane M_7, forms the central portion of the axial fibre and will be discussed more fully below. The whole of this fibrillar system is surrounded by the membranes m_0 and M_5. The compact central fibril bundle is seen quite clearly in Plate IV b as a dark line. The membrane m_0 is visible in Plate IV a. A doublet coil C_5C_5' frequently referred to as the cortical helix is closely wound round M_5. Each coil is 25–30 mμ wide, with a separation of 15 mμ. They make an angle of $85^\circ \pm 5^\circ$ with the long axis of the tail. A diagrammatic section of the main-piece is given in Fig. 4 a–4 c.

E. 3. The Axial Fibre. The detailed constitution of the axial fibre is an important and complex problem. We have come to the provisional conclusion that the central compact bundle remains intact throughout the length of the tail. Twisting of the tail may make it appear to one side (Plates IV b and IV c). Arranged loosely round it are the remaining six fibrils, the whole (see Fig. 4 c) being enclosed in the membrane m_0.[1] Each of the two outer bundles is composed of three fibrils; the central compact bundle, however, consists of *three pairs* of fibrils (Plate IV c). There are thus twelve *fibrils* in all. The evidence for this conclusion is based on numerous observations of brush-effects and the examination of fibrous strands at tail-breaks. The constituent filaments have already been mentioned as a feature of the tail-end which is, in fact, only a protruding portion of the slightly modified axial fibre. Our present view is that each of the twelve fibrils consists of a doublet (i.e. two *filaments*) and that each doublet is enveloped by two-ply helix, $C_{13}C_{13}'$,[2] (Plate IV b). The axial fibre may therefore be said to consist of 24 filaments. As each helix seems to be a double or two-ply coil it is seen that there are really 48 filaments in this structure. There are indications that there is a membrane, M_{13}, there is between the filament doublet and C_{13}, but more evidence is required to make sure that this is not an artifact (cf. Plate III d).

E. 4. The Middle-Piece. This portion of the sperm lies between the tail-piece and the neck, and is distinct from the tail-piece in several particulars: (i) It is thicker (~ 800 mμ) and much shorter (~ 14 μ). (ii) It is more complex. (iii) Photographs of live ram sperm taken in visible light between crossed Nicols (Plate VIII a, b, c) show that the middle-piece is the only

[1] Trypsin digestion removes this.

[2] Similar coils are found round the fibrils in the neck area. See Plate VI a (later text), and Plates III d, VIII a, and VI d, where fragment of neck with one clear fibril is visible.

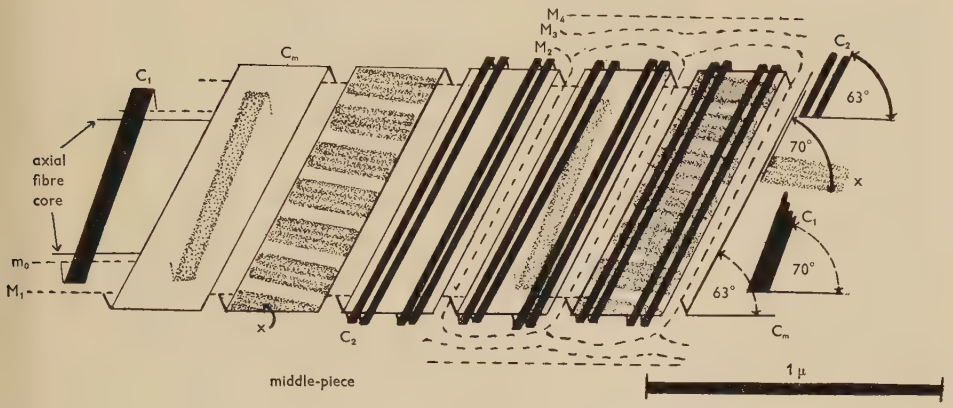


Fig. 5 a.

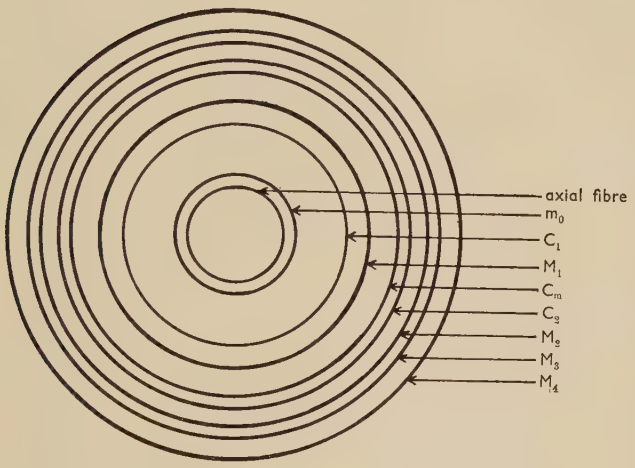


Fig. 5 b.

Fig. 5. Structure of the middle-piece. a. Longitudinal section. b. Transverse section.

part of the whole sperm which appears to possess 'structure', although the head shows marked birefringence and orientation of its contents. The optical observations of the living sperm are of great value for comparison purposes.

Fig. 5 a illustrates the construction of the middle-piece, the complexity increasing from left to right. A purely diagrammatic view of the middle-piece seen in section is given in Fig. 5 b. The helix C_1, about 60 mμ thick, was exposed by trypsin treatment (Plate VII c and d) and is found to envelop

the axial fibre. C_1 is surrounded by the membrane M_1, which in turn is surrounded by the membranous band C_m. Detailed examination has shown that C_m really consists of two bands, equally spaced, coiled side by side; cf. Plate IV d and VI d. The close proximity of these two bands, brought out clearly on drying, inherent in electron microscope technique, no doubt led to the use of the term *mitochondrial sheath* in the early work. There is no indication from these experiments of the location of the original mitochondria. Cross striations shown in fig. 5 a cross brackets C_m at an angle of $110° \pm 5°$ to the band axis; these double striations are 55 ± 5 mμ wide and are shown in Plates V b and IV d. The coil system C_2C_2' can be seen in Plate V a and is wound from a double strand. M_2, Plate V d as shown in Fig. 5 a is perhaps misleading. It is not another band like C_m but, rather, a continuous sheath which is twisted in such a way as clearly to follow the contour of C_m. M_2 and other membranes of the middle-piece receive further consideration below.

TABLE I

Measurements of Mitochondrial Sheath or Middle-piece Coil Cm in Ram Sperm.

	Diameter of Sheath (μ)	Characteristic Angle of Coil Θ in degrees	Pitch of Coil p (μ)	Width of Cm	Length of Middle-piece (μ)
Light Photographs of Live Sperm	1.0	71	0.4	0.33	12.6—14
Electron Micrographs	0.8—1.0	60 ± 8[1]		0.34	

Measurements on this sheath or coil both from electron micrographs and polarized light photographs are given in Table I. Too much stress must not be placed on the absolute values obtained by either method. The measurements of pitch of coil and width of sheath obtained in the light microscope are subject to diffraction errors; and the electron micrograph measurements are dependent on the accuracy of calibration of the instrument, which cannot be taken as better than 5 per cent. The figures are nevertheless interesting in that no gross shrinkages are present as a result of the vacuum drying process to which an electron microscope specimen is subjected. In drawing Fig. 5 a as a representation of the structure of the middle-piece sheath and coil C_1, we have taken the angles as 63° and 70° respectively.

The posterior termination of the middle-piece is in Jensen's ring, which is 200 ± 50 mμ thick (Plate IV a). The anterior termination is not so clear (Plate VI a and V b) and this lack of clarity is possibly connected with

[1] This represents the range of values, not an estimate of accuracy.

minor effects of evacuation treatment. Some electron micrographs have suggested a smooth, apparently undifferentiated, region in the middle-piece below the neck-junction (Plate V c). There is no doubt, however, from the light photographs of Plate VIII that the mitochondrial sheath (C_m of Fig. 5 a) still exists at not more than 0.4 μ from the base of the head, and this is confirmed by Plate VI c. Investigation of the middle-piece has been specially fruitful in revealing membrane systems pertaining not only to this part of the sperm but to underlying regions. For example, in Plate VI a and Plate V c can be distinguished the membrane M_1 which underlies the mitochondrial sheath. Membrane M_2 (cf. Plate V d) can just be distinguished on Plate VI b.

Membranes are not easy to demonstrate in electron micrographs[1], especially when they lie over or under much denser material. Examination of the plates also shows that the presence of a membrane has sometimes to be inferred from the slight projections of less dense material beyond a major boundary. Special care has to be taken to allow for the effect due to a membrane lying on top of material of varying thickness. On subsequent drying and shadowing this differential sagging is apt to be misleading in the sense of suggesting additional membranes. For these reasons, and because we believe that further work will clarify some of these points, the membrane systems described in this paper are put forward tentatively. Fig. 5 b shows a diagrammatic section of the middle-piece.

E. 5. The Neck. The neck is a complex structure joining the head and flagellum and consequently lying between the posterior portion of the head and the anterior portion of the middle-piece. The junction is mechanically the weakest part of the cell. The neck itself has a complexity of its own superposed on structural features already established for the middle-piece. The work of Marza (11) suggests that this part of the sperm is chemically the most complex.

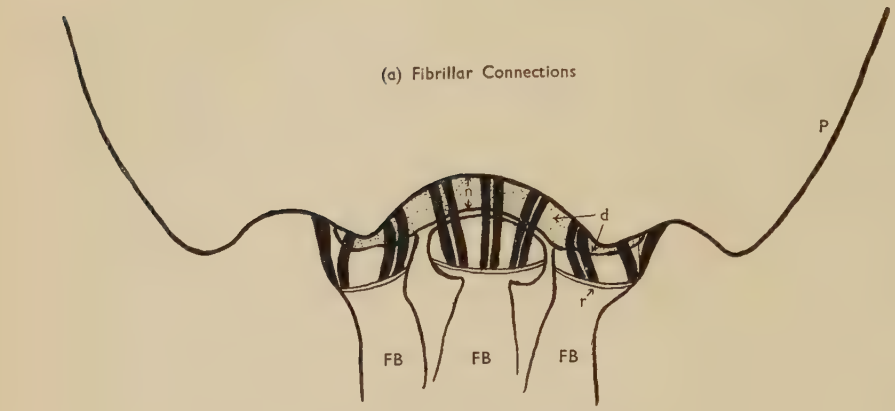
Three types of linkage between head and neck are found. They are:

(a) Fibrillar
(b) Fibrous and membranous
(c) Coil connections.

While this classification is rather artificial, it makes it easier to visualise the structures involved. Fig. 6 a shows some of the fibrils which extend across the neck-gap 'n'. These are visible on Plate VI c and Plate III b. Various electron micrographs have confirmed that these fibrils are continuous from points inside the head through the neck to the middle-piece. The number

[1] The disembodied membrane M_2 of Plate V d is quite exceptional.

Fig. 6 a.



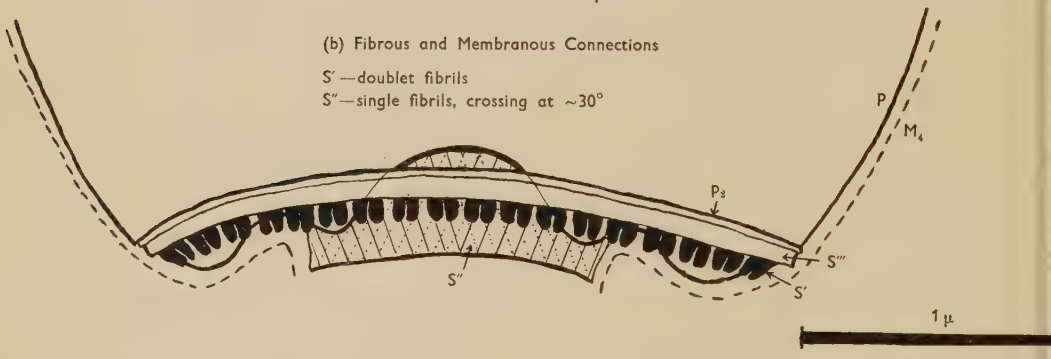


Fig. 6 b.

Fig. 6. Structure of the Neck. a. Fibrillar. b. Fibrous and membranous. c. Coil connections.

of fibrils involved is not clear but is considerably greater than appears in Fig. 6 a. It is reasonable to assume that these fibrils are an unorganised picture of the fibril bundles of the middle-piece and flagellum. The ring r is a thickening round each fibril bundle and appears to be superficial only, cf. Plate VI a.[1]

Fig. 6 b is largely self-explanatory. The pictorial value of the diagram is stressed rather than quantitative accuracy, particularly in the case of S′. Briefly, there are two cuffs, S‴ and S′, below the posterior edge, p_2, of the post-nuclear cap. S‴ is membranous, while S′ consists of doublet fibrils, as seen in Plates II b, II a and III a and III b. Their fibrillar counterpart

[1] The general fibrous nature of the head-neck junction may also be inferred from Plate VI b where a particularly long fibril remains still attached to the neck on the far side.

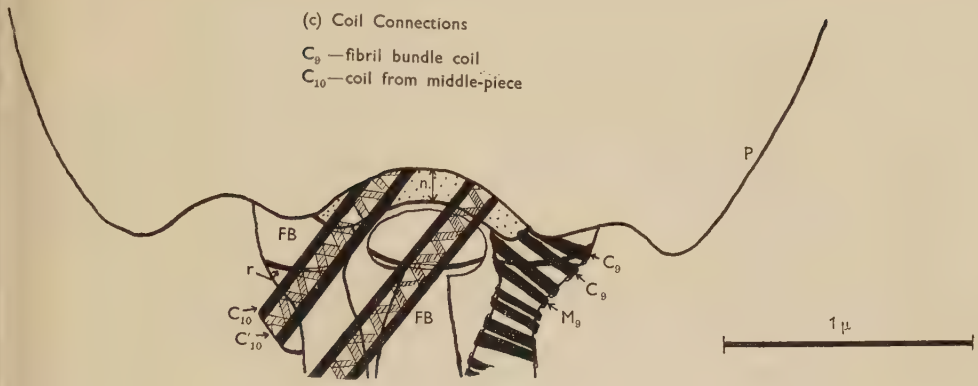


Fig. 6 c.

in the middle-piece remains unknown at present. An altogether different kind of cuff can be seen in S″. These are single fibrils, cf. Plate II a.

Fig. 6 c deals with coils. C_9 represents the doublet coil surrounding the fibril bundle. This can be seen quite well on the right-hand of Plate VI a, and very clearly on Plates III a and VII a. A flat coil $C_{10}C_{10}'$ goes right into the head. It may be a continuation of C_m and can be seen on Plate VI c.

F. OPTICAL MEASUREMENTS

Polarized light (λ 5.000 A) photographs with crossed Nicols of live ram sperm ejaculated a few hours previously show a number of interesting features, most of which require further detailed study outside the limits of the present paper. As there is no doubt that optical measurements have a distinct and useful bearing on the conclusions to be drawn from electron micrographs some of the preliminary results will be mentioned here. It is already well-known from the work of Schmidt that sperm-heads are strongly birefringent as a result of the highly oriented desoxyribose nucleic acid content. In the case of *Sepia*, Schmidt has deduced that the axes of the nucleic acid chains lie roughly parallel to the major axis of the sperm-head. Transmission of light by oriented nucleic acid molecules is greatest when the electric vector is parallel to the planes of the purine and pyrimidine components; these lie perpendicular to the chain direction. (For this reason the birefringence with respect to the long axis of the molecule is negative; that is to say, the refractive index for the electric vector parallel to the long axis

of the chain is smaller than the refractive index corresponding to the electric vector at right angles to the chain.)

The polarization microscope photographs of Plate VIII a, b, c, were obtained with a water-immersion objective of 1.2 N.A., and the resolving power of the instrument was approximately $\lambda/2$ (~ 0.25 μ). The finest bright line-object would thus give a central diffraction maximum of total width ~ 0.25 μ and it is thus impossible to be certain of the size of any objects less than this.

The photographs obtained between crossed nicols indicate little or no birefringence for light passing through the head along the c axis shown in Fig. 7, where the axes a, b, and c are mutually perpendicular. If, however, the light is parallel to the b-axis (i.e. the head is "edge-on" to the observer), there is birefringence, and between crossed nicols a sharp image then appears as a two-line diffraction pattern, as shown in Plate VIII a. From the distance apart of these lines it may be safely inferred that the mean thickness of the head is not much greater than 0.5 μ and very unlikely to be greater than 1 μ. The length of the head, measured as shown in Fig. 7 and Plate VIII c is 8.4 μ and the maximal width 4.3 μ.

From the measurements of the sign of birefringence we may also deduce that in ram sperm the direction of the nucleic acid columns is parallel to the short c-axis of Fig. 7 a and b.

Further study of the sperm-heads viewed broadside-on as in Fig. 7 a indicate

(a) the equatorial region so clearly seen in the electron micrographs, ultraviolet and phase-contrast pictures.

(b) the acrosome and acrosome cap.

(c) strong indications of a peripheral boundary or framework. The substantiation of this feature by further work would clearly be of great interest as a determining factor in the shape of the head and its remarkable resistance to mechanical disintegration.

(d) A number of photographs show quite clearly three sets of neck fibrils. This is in full agreement with the findings of the electron microscope. At the extreme anterior position the bundles are approximately 0.5 μ apart.

(e) The head (broadside-on) frequently shows a granular structure and there are suggestions in some photographs of the complex crab-shaped structure quite frequently seen in the electron micrographs (Plate III c).

(f) Information about the middle-piece is also of importance from several points of view. The length is ~ 14 μ; this compares satisfactorily with the electron microscope measurements. The most striking feature of the photographs is the visibility of the turns of the mitochondrial helix, C_m (Plate

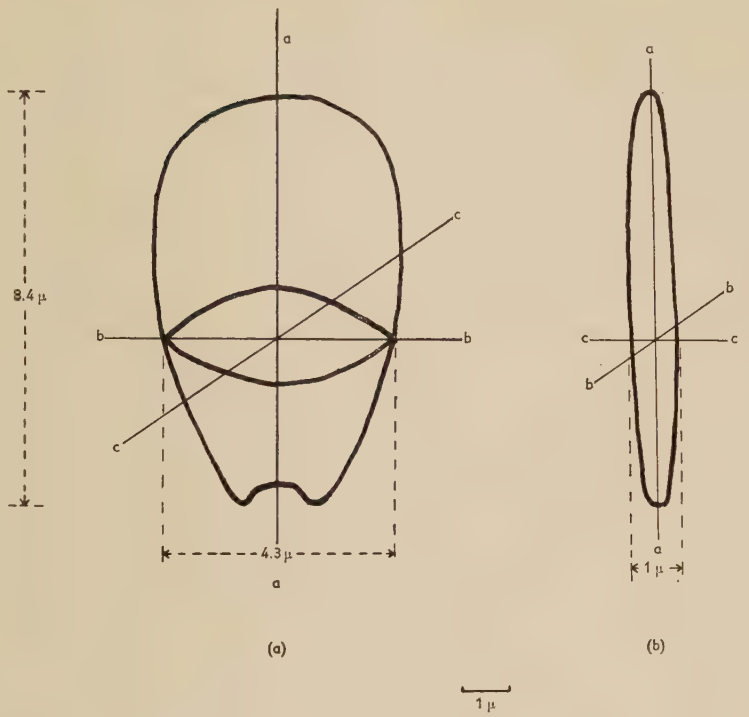


Fig. 7. Head symmetry. a. Broadside-on position. b. Edge-on position.

VIII b). Measurement of the acute angle Θ these turns make with the axis indicates a mean value of ~70°. The corresponding angle on the electron micrographs is 60°±8° (see Table I, page 18).

On all polarized light photographs the axial fibre system shows up as a dark line. Investigations to decide whether this implies a difference in nature between the fibre and the mitochondrial sheath, or simply a difference in orientation, are in progress.

An ultraviolet (λ 2,650) photograph is included in Plate VIII d. At this stage we do not wish to stress the features of the ultraviolet photograph as necessarily indicating differential absorption in various parts of the sperm. Such differences may well arise from refraction properties and careful work is required to decide such points. The ultraviolet photograph shows a region below the acrosome at A (Plate VIII d) which corresponds roughly to the region shown on Plate II d and seems too wide to be simply the boundary of the acrosome cap A_1. The equatorial segment e and the mitochondrial coil C_m at C can also be seen in the ultraviolet photographs.

G. DISCUSSION

G (a). The Cross-Section of the Sperm. It has been possible to come to some conclusion about the cross-section of various parts of the sperm. The evidence is as yet mostly indirect.

(i) *Head.* It has not been possible so far to obtain a profile view of the sperm-head of ram in the electron microscope. The most reliable evidence has been obtained from polarized light pictures taken between crossed nicols and discussed in Section F to which reference should be made for dimensions.

(ii) *Neck and Middle-Piece.* While the neck is still flattened there is little doubt that the cross-section of the middle-piece is very nearly circular, and of radius 0.4 μ. (The electron microscope evidence for this is good, and there is direct confirmation from observations on living sperm in the light microscope.)

(iii) *Main-Piece.* This is flattened and has an elliptical cross-section. Dimensions for this part of the living sperm are difficult to obtain. A great deal of flattening in the electron microscope is undoubtedly due to drying effects. There is no reason to suppose that the axial fibre itself is not essentially cylindrical. The flatness of the dried tail, however, is confirmed by its appearance when passing over obstructions, or when twisting occurs.

(iv) *The Tail-End* has a circular cross-section, or very nearly so. This is consistent with the close-packing observed in that portion of the flagellum, and the fact that all fibril bundles and doublets have split up into an assembly of uniform fibril components.

G (b) Electron Micrograph Background. Globules and rods have been observed in the background and are characteristic of the treatment used. In general their appearance may be ascribed to a loss of surface structure and it is notable that the globules or rods appear in the vicinity of the part in question. It has occasionally been possible to trace the origin of background matter. Thus in the case of trypsin-treated specimens the rod-like particles are undoubtedly in part due to the dissolved mitochondrial sheath C_m (cf. Plate VII c).

Flake-like irregularly shaped particles have also been observed, and fibrils probably originating in some reticulum structure of the head are frequently to be seen.

G (c). The Skeleton. A number of observations suggest that the ram sperm-head may have a skeletal structure roughly coincident with the boundary

of the head as seen in Fig. 2. (i) The head is difficult to rupture along this periphery. Plate VII c indicates how ultrasonically produced rupture stops short of the boundary. (ii) A number of electron micrographs of unshadowed preparations have indicated an almost opaque portion round the base and upwards along each edge to a little beyond the equatorial segment. Since this effect is not apparent in shadowed preparations it must be caused by denser material rather than thickening. (iii) Measurements of width of the head from electron micrographs show it to be independent of even drastic treatments such as that of C. 5 (shaker method) which results in the partial emptying of the head. (iv) Polarization microscope photographs of sperm-heads taken broadside-on (as viewed in Fig. 7) between crossed nicols show a boundary region distinct from the rest of the head (cf. Plate VIII c).

G (d) The Work of Bretschneider on Bull Sperm. The evident similarity of ram and bull sperm in all major features makes necessary a critical examination of Bretschneider's results (3) for bull sperm. Papers III and IV only will be considered as they represent a revised interpretation of structures indicated in his earlier papers. The fact that Bretschneider deals primarily with unshadowed preparations may have caused him to overlook some of the finer detail. It may be as well to state once more that it is quite essential to use shadowed and unshadowed preparations side by side as the evidence obtained is frequently complementary. This is brought out particularly in the detail and interpretation of head structures and middle-piece. Our techniques have, on the whole, been simpler, but we have shown that there is in addition to an equatorial zone a sharply defined equatorial segment. The zone is due to local differences in density of the head while the segment is a strictly surface phenomenon. With regard to the identification of chromosomes present in the sperm head, it seems to us that Bretschneider's interpretation based on his silver technique does not eliminate the possibility of artifacts.

We differ chiefly on the interpretation of neck, middle-piece and tail structures.

Tail-end. We have found no particular tendency to disintegration in this region and have observed not only a doublet coil surrounding it, but in addition, evidence of the protoplasmic sheath. While the tail-end is no doubt capable of rudder action with respect to the rest of the tail, it is by no means always bent away from the tail. The cortical helix of the tail-piece of ram sperm is definitely a doublet, not a triplet; and we have confirmed this in an examination of bull sperm. This doublet is not identical with the tail-end coil which is different in density and dimensions.

Middle-piece. The complexity of the middle-piece structure as described by us explains why Bretschneider appeared undecided about the doublet or triplet nature of the middle-piece coils[1] and, in fact, whether the helix was genuine. It is possible to get pictures where (C_2C_2') ($C_2C_2' + C_1$) or C_m only are prominent. Bretschneider's suggestion that, what we refer to as the post-nuclear cap, the "neck-band", and the outer middle-piece membrane represent a continuous membrane is erroneous. The confusion here is due to insufficient identification of the various parts.

The neck-structure for ram sperm was found to be far more complex than the corresponding structure described by Bretschneider for bull sperm. We have found no evidence which would support his picture of the axial subfibril arrangement (cf. paper IV, p. 530) or for the claim that fibrils of three different widths are involved. Nor is there any reason to suppose that the apparent arrangement of individual fibrils in bundles is an artifact. Rather, it is an essential characteristic of the undamaged sperm. While there are indications of granules in the neck structure they do not, as Bretschneider contends, represent the origin of the fibrils concerned. We have shown that such fibrils and their finer sub-units form part of the head structure. The claim that there is some protein-binding substance between head-base and neck which may, on drying, be pulled out into threads does not account for the coarser fibrillar junction we have observed. There is evidence that continuity of the middle-piece fibrils is maintained both in inclination and dimensions across the neck gap. According to Bretschneider there is a helical structure in the neck which disappears on lysis except where it adheres to the axial fibre. In ram sperm there is convincing evidence that this "debris" adhering to the fibrils is genuine (cf. Plate VI a and VI c) and represents doublet coils which appear in modified form in any brush effect which exposes individual fibrils (Plate III d). Both the coil and the fibrils decrease in diameter as their distance from the neck increases. The suggestion made in Bretschneider's earlier paper (I) that the fibrils give rise to ~ 5 subunits is, in our opinion, based on insufficient evidence. There is every probability that the fundamental doublet sub-structure of individual fibrils also holds for bull sperm.

It is true that a helical structure can be seen in the neck (cf. our Plate VI c); from its appearance, however, it would seem to be a continuation of C_m and confusion with the fibril doublet-coil is thus impossible.

[1] In Bretschneider's papers the middle-piece coils are referred to as doublets (Paper I); triplets (Paper III); as only a thickening in the enveloping membrane (Paper IV).

H. SUMMARY

The microstructure of the mature ram spermatozoon has been investigated by means of the electron microscope, and has been supplemented by preliminary optical studies. Various fixatives and stains were employed, and extensive use was made of ultrasonics as a means of partial disintegration. The results indicate a more complex structure than has yet been found for other mammalian spermatozoa.

The investigation included an examination of the membrane system of the head, and various features such as the equatorial segment, the acrosome cap, the innermost membrane and structures underlying it. The evidence for a skeletal framework of the sperm-head was also discussed.

The axial-fibre was found to consist of 12 fibrils, each of which is made up of two filaments surrounded by a doublet coil. The suggestion was put forward that a fibril bundle consisting of 3 pairs of fibrils runs down the whole length of the tail. The main-piece itself was shown to be surrounded by a doublet helix; the tail-end consisted of modified fibrils of the axial-fibre, surrounded by a fine doublet-helix of its own.

Considerable attention was given to the middle-piece structure where three systems of helices were identified.

The complexity of the neck structure was investigated; the junction of the head and middle-piece was found to be partly fibrillar, and partly fibrous and membranous. The continuity of the fibrils from the middle-piece and the fibrils which disappear into the head was established.

Provisional membrane systems were described for middle-piece, tail, and tail-end.

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APPENDIX ON TERMINOLOGY

HEAD

G — Galea capitis
A_1 — Acrosome cap
A_2 — Acrosome
I — Intermediate membrane or system of membranes
N — Surface membrane of nucleus
P — Post-nuclear cap
p_1 — Anterior edge of P (serrated)
p_2 — Posterior edge of P (frequently referred to as "basal-ring" or "neck-band")
FB — Fibril bundle
n — Neck-gap or spacing (the word gap is used figuratively only)
S′ — Outer cuff
S″ — Inner cuff
S‴ — Intermediate cuff
U — Position of initial ultrasonic puncture
(c) — Position of centriole
e — Equatorial segment
E — Nuclear ring
R — Arc-like thickening inside head
M_4 — External head membrane, generally missing, and probably the lipoid capsule

NECK

C_{10} — Band coil round neck, originating in middle-piece
FB — Fibril bundles
C_9 — Coils rounds FB's
d — Doublet fibrils, bridging gap between head base and FB's
r — Ring (membrane boundary?) on FB's
M_7 — Membrane round each FB (not shown in Fig. 6 c)

MIDDLE-PIECE and TAIL

m_0 — Membrane surrounding axial fibre
C_1 — Thick helix outside m_0
M_1 — Membrane outside C_1
Cm — Membranous band
X — Cross-striations found on it
M_2 — Membranous sleeve
M_4 — Further membrane, probably lipoid capsule
C_2 — Doublet coil superimposed on Cm
J — Jensen's ring (distal centriole)
M_5 — Tail-piece membrane
C_5 — Doublet coil wound on M_5
M_6 — Tail-end membrane
C_6 — Coil surrounding tail-end
F — Individual axial fibrils
f — Individual filaments making up one fibril
M_{13} — Membrane surrounding fine filaments
C_{13} — Doublet coil round M_{13}
M_{12} — Membrane enveloping fibrils in FB (neck area)
C_{12} — Doublet coil round it
M_{14} — Outer membrane

N. B. Although dimensions of individual fibrils seen in the neck and in the tail are very different, the actual structure appears to be the same, which indicates that presumably

$$M_{12} = M_{13}$$
and $$C_{12} = C_{13}$$

both membrane and coils having undergone certain minor modifications.

TEXTS TO THE PLATES

PLATE I a. Sheep sperm-head showing fibrous galea capitis, sharply defined equatorial segment, and post-nuclear cap with its posterior boundary in the neck area. The fibrous attachment of the neck is just visible as is the doublet nature of the neck coil. Fixed in formalin and stained with phosphotungstic acid for 3 hours.
Magnification × 12,300.

PLATE I b. Sheep sperm-head showing complex fibrils and globular material in galea capitis. Fixed with formalin and stained with osmium tetroxide.
Magnification × 11,800.

PLATE I c. Anterior half of head, showing galea capitis. Fibrils and globules are visible (cf. text).
Fixed with formalin and stained with phosphotungstic acid. Shadowed.
Magnification × 22,100.

PLATE I d. Galea capitis, showing complexity of fine fibrillar network. Also, various types of globule.
Fixed in formalin and stained with phosphotungstic acid.
Magnification × 12,100.

PLATE II a. Sheep sperm-head showing acrosome cap. Note that the equatorial segment covering is missing. U is the puncture which appears first on application of ultrasonic treatment. The fibrous nature of the cuff at the base of the head can also be seen in this picture. Fixation: osmium tetroxide vapour; treated with ultrasonics (26 watts/cm²).
Magnification × 11,600.

PLATE II b. Head showing bared upper portion where the acrosome cap and underlying acrosome are missing. At the base, the doublet cuff, S′, is visible. Fixed with osmium tetroxide vapour, and treated with ultrasonics (26 watts/cm²). Shadowed.
Magnification × 11,600.

PLATE II c. Head, with galea capitis. Fibrils are concentrated in equatorial segment. The post-nuclear cap is clearly visible, as is the fibrillar nature of its serrated edge.
Fixed with formalin and stained with osmic acid.
Magnification × 9,200.

PLATE II d. Head, showing characteristic structures protruding near the tip; also the split membranes. Overlying tail shows M_0. Fixed with formalin and stained with osmic acid.
Magnification × 13,300.

PLATE III a. Sheep sperm-head showing acrosome cap and dense portion R lying immediately below the acrosome surface. Note also the "neck-gap," outer cuff, and the coil around one of the neck fibril bundles. The middle-piece of another sperm lying on the left of the picture shows bands Cm with cross-striations X well defined. Note fibrils and globules in equatorial segment and beyond it.
Fixed with formalin and stained with osmic acid.
Magnification × 13,800.

PLATE III b. Head, without galea capitis, showing characteristic structure near the tip. The neck gap is clearly visible, with fibrils bridging it. p_2, the posterior boundary of the post-nuclear cap, is seen running across the neck. The three fibril bundles are quite distinct.
Fixed with osmium tetroxide vapour.
Magnification × 11,600.

PLATE III c. Head, showing characteristic crab-like structure.
Fixed with formalin and stained with osmium tetroxide.
Magnification × 12,200.

PLATE III d. Middle-piece brush-effect, showing 3 double fibrils. Arrows indicate portions where coils surrounding the fibrils are visible. A detached neck-portion can be seen in the lower half of the picture, with one fibril attached, showing surrounding doublet coil.
Fixed with formalin and treated with ultrasonics.
Magnification × 13,000.

PLATE IV a. Strands of extended fibrils, bridging gaps caused by breaks in the tail. Jensen's ring is visible as indicated. Osmium tetroxide fixation. Subjected to ultrasonics (26 watts/cm²).
Magnification × 11,600.

PLATE IV b. Brush-effect showing 6 loose fibrils plus two derived from the tail-coil doublet, together with six more from one fibril bundle. The fibril coils are visible. Fixation: Osmium tetroxide vapour.
Magnification × 20,000.

PLATE IV c. Tail-end brush-effect and tail. Tail-end shows membrane M_6 and surrounding helix. Neighbouring tail shows thicker portion due to fibril bundle.
Fixed with osmium tetroxide vapour.
Magnification × 19,300.

PLATE IV d. Sheep sperm: two typical middle-pieces. (Ignore the tail lying to the right of middle-piece I.) I shows a flat band structure, while II draws attention to membrane M_1 (fig. 5) and the superposed double-band system, Cm.
Fixation: formalin, and stained with phosphotungstic acid.
Magnification × 24,000.

PLATE V a. Typical neck and middle-piece. The arrow marks the doublet coil C_2C_2' of fig. 5 a which is superposed on the mitochondrial sheath. The inner and outer cuffs at the base of the head should be noted in one of the specimens. Fixation: formalin and stained with 0.2 per cent osmium tetroxide solution for 30 minutes.
Magnification × 9,200.

PLATE V b. (The numerals indicate different appearances of middle-piece.)
Neck (1) notable for showing structure corresponding to centriole and slightly denser upper region of middle-piece. The mitochondrial sheath Cm is prominent and the membrane M_2 is seen to cover Cm and extend beyond it to the base of the head.
Fixation: osmium tetroxide; subjected to ultrasonics 26 watts/cm².
Magnification × 11,600.

PLATE V c. *A* shows a membrane surrounding the compact central middle-piece core. At *B* are to be seen a number of constituent fibrils and a loosened mitochondrial sheath. Formalin fixed; treated with ultrasonics.
Magnification × 14,000.

PLATE V d. Middle-piece membrane M_2. This membrane normally encloses the mitochondrial sheath but is here seen empty. Undiluted sperm fixed in osmic acid vapour, diluted and treated with ultrasonics (26 watts/cm²).
Magnification × 12,600.

PLATE VI a. Neck and middle-piece, showing mitochondrial sheath Cm and membrane M_1 underlying it. The ultrasonic treatment has exposed constituent fibrils in the neck. Each fibril is surrounded by a doublet coil, C_{12}. The terminating ring r (cf. Fig. 6 a) is visible. Ultrasonics (25 watts/cm²) applied to a live sperm suspension in tyrode. Afterwards fixed with formalin and washed.
Magnification × 14,200.

PLATE VI b. Middle-piece showing fibril extending beyond neck boundary. The cross-striations on Cm (X on Fig. 5 a) are clearly visible. Also the general twist of the fibrils in the upper region of the neck.
Ultrasonics (25 watts/cm²) used on live sperm suspension in Tyrode.
Magnification × 14,800.

PLATE VI c. Head-neck junction showing
1. Fibrillar junction across neck-gap.
2. Doublet-cuff.
3. Neck helix $C_{10}C_{10}'$.
4. p_2.
M_2 is also visible.
Fixed in formalin.
Magnification × 17,000.

PLATE VI d. Brush-effects, mostly of middle-pieces. Doublet coils C_{12} (marked b on the plate) surrounding the filaments are visible (cf. fig. 4, 7) and also the underlying membrane M_{12} (marked a on the plate). Mild application of Mann's shaker method.
Magnification × 15,200.

PLATE VII a. Two necks, A and B, showing rings r. B illustrates doublet coil C_9 surrounding fibril bundles. Also evidence of M_1 and M_2. Note fibrillar upper edge at A.
Ultrasonics applied to live sperm suspended in Tyrode. Fixed in formalin after treatment, and prior to washing.
Magnification × 12,800.

PLATE VII b. Post-nuclear cap missing from head, while preserved at the edges, outlining what would correspond to the darkened skeleton-like portion of unshadowed preparations. Preparation as for VII a.
Magnification × 13,200.

PLATE VII c. Partially digested neck and middle-piece, showing C_1, while Cm is missing. The central fibril bundle shows indications of C_9. The background is typical for this kind of preparation.
Tyrode-diluted sperm suspension was digested with trypsin for 72 hours at room temperature.
Magnification × 21,500.

PLATE VII d. Partially digested neck and middle-piece. The doublet nature of the fibrils is evident as they approach the upper boundary of the neck. C_1 is well-preserved over considerable stretches of middle-piece.
Tyrode-diluted sperm suspension was digested with trypsin for 72 hours at room temperature.
Magnification × 12,200.

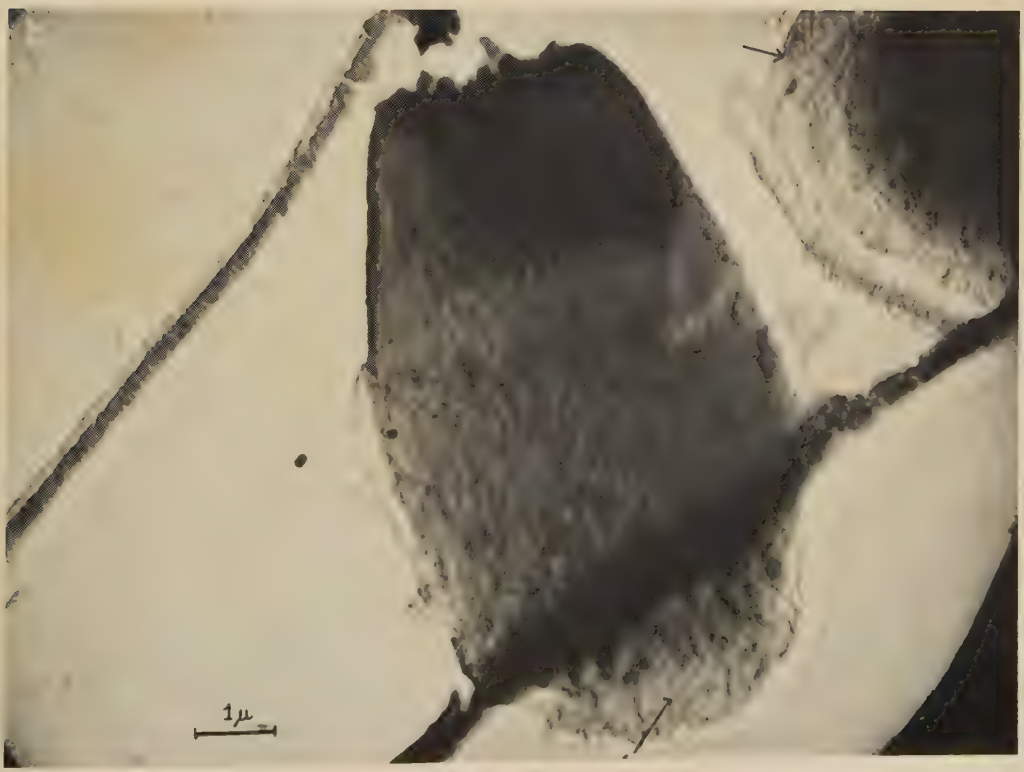
PLATE VIII a. Diffraction pattern produced by ram sperm head viewed edge-on (cf. Fig. 7 b) between crossed nicols (λ 5,000).
Magnification × 2,400.

PLATE VIII b. Live ram sperm viewed between crossed nicols showing helical nature of middle-piece.
Magnification × 2,400.

PLATE VIII c. Live ram sperm viewed between crossed Nicols. Note particularly the three neck-fibrils and apparent skeletal boundary of head.
Magnification × 2,400.

PLATE VIII d. Ultraviolet (λ 2.650) photograph of ram sperm in which helical structure of middle-piece can just be distinguished at C, together with the equatorial segment and the region at A which corresponds roughly with the dense portion to be seen in Plate II d.
Magnification × 1,050.

a
1μ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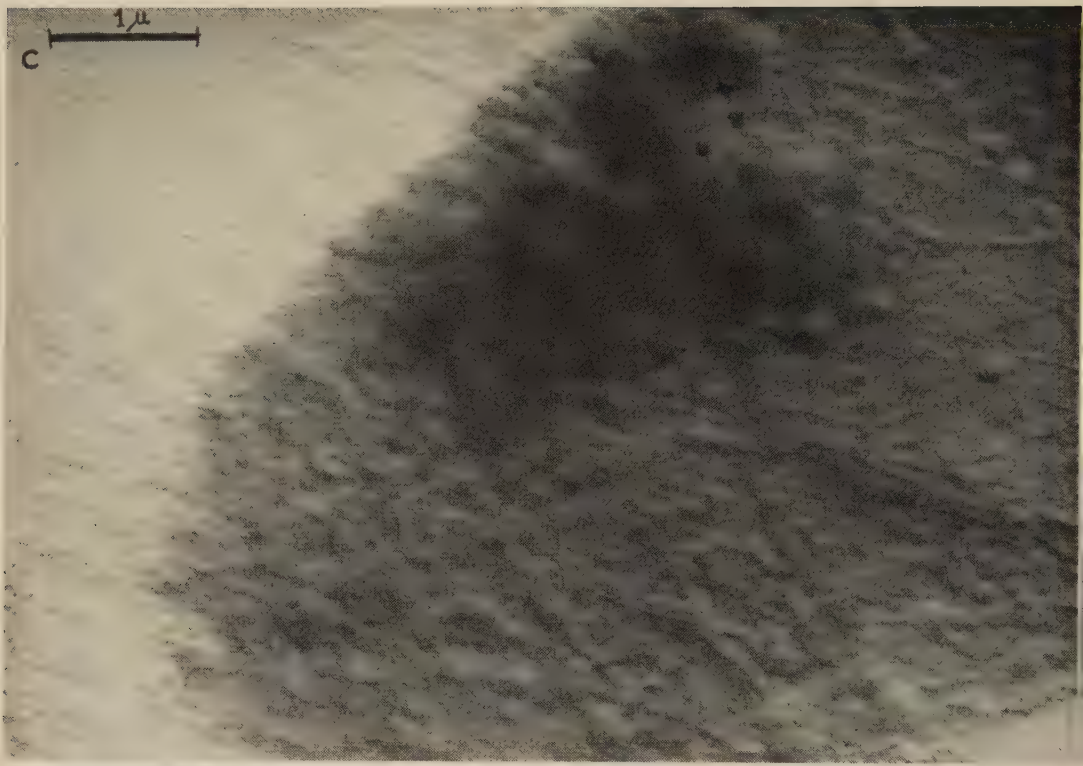
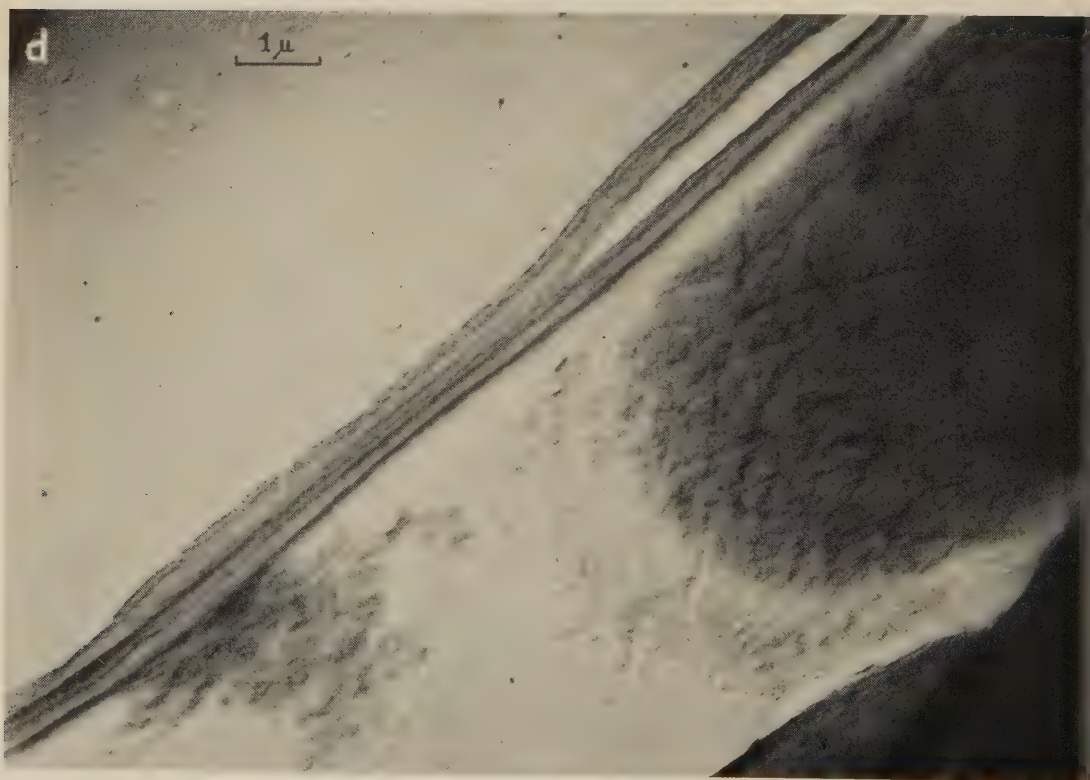
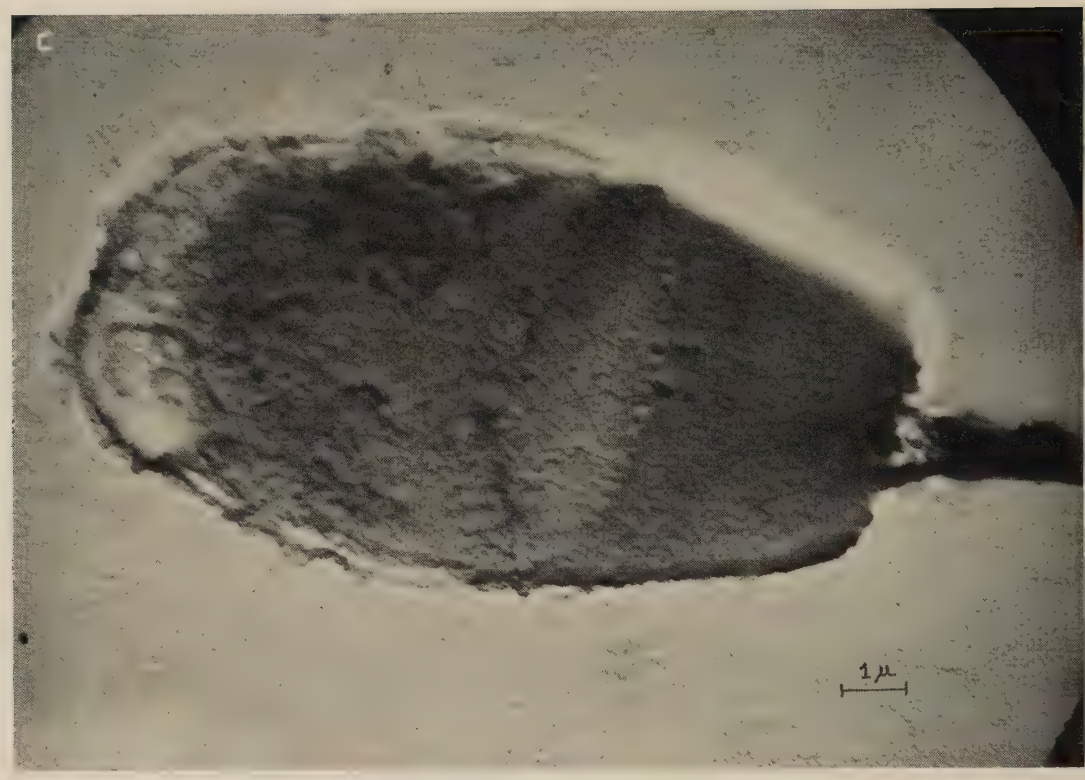
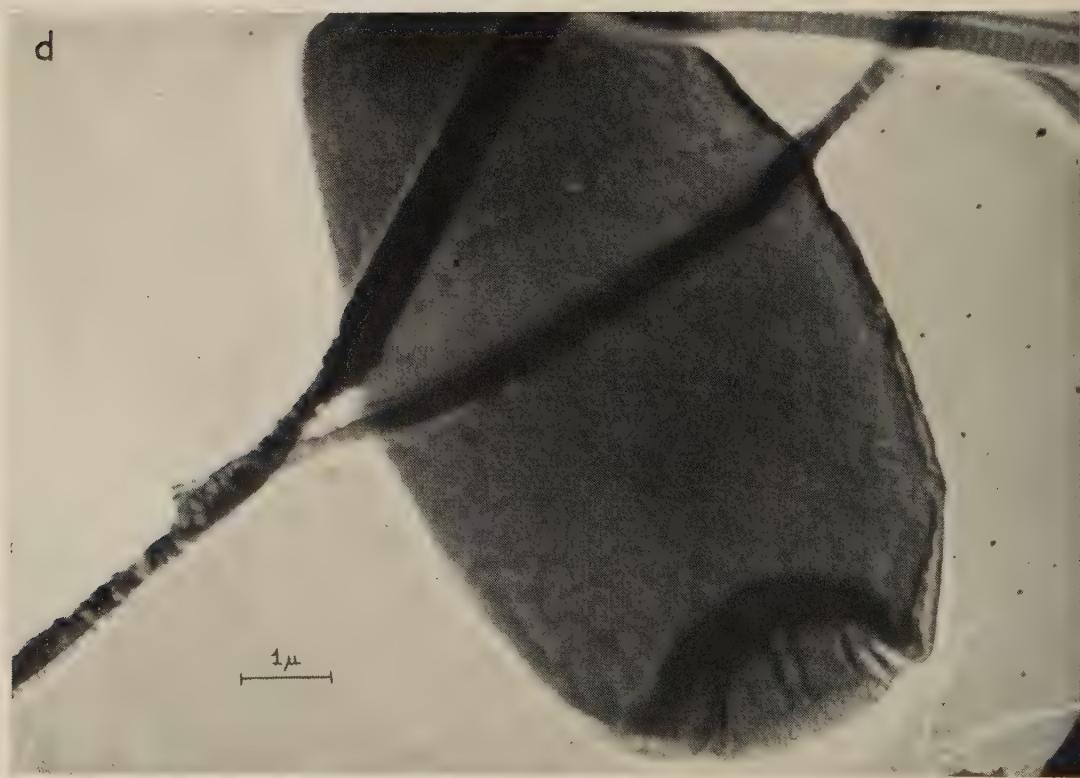

1μ
c


1μ
d

a
1μ

b
1μ

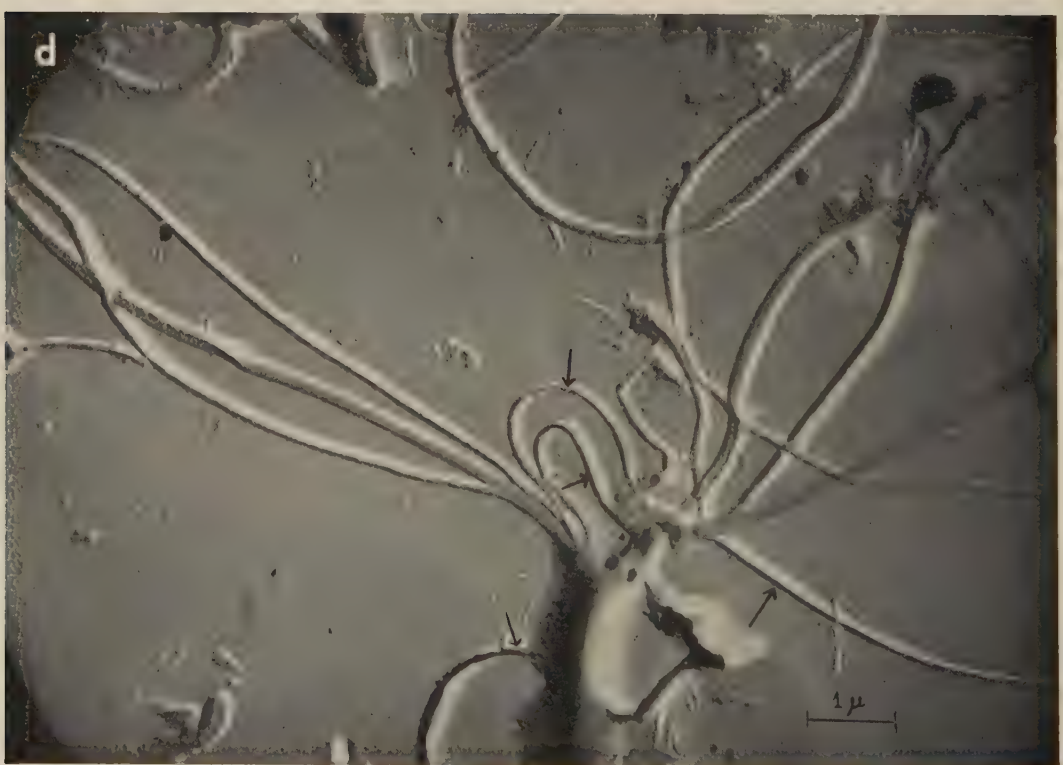

c
1μ


d
1μ

a
1 μ

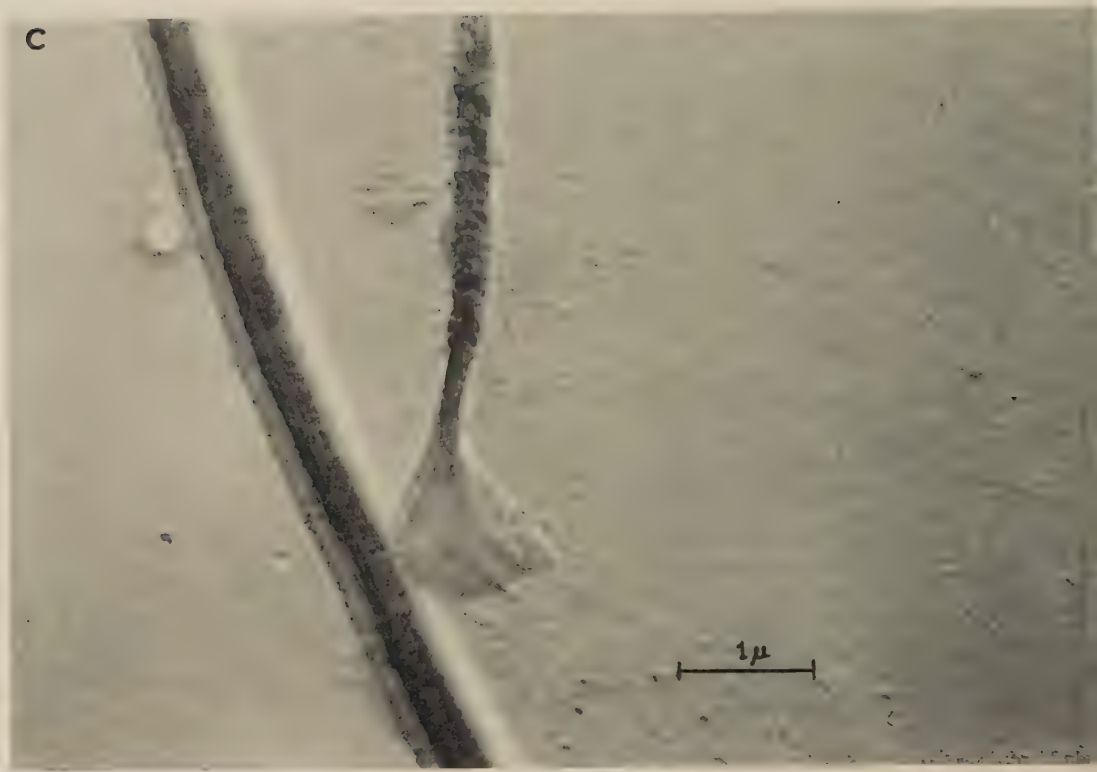
b
1 μ

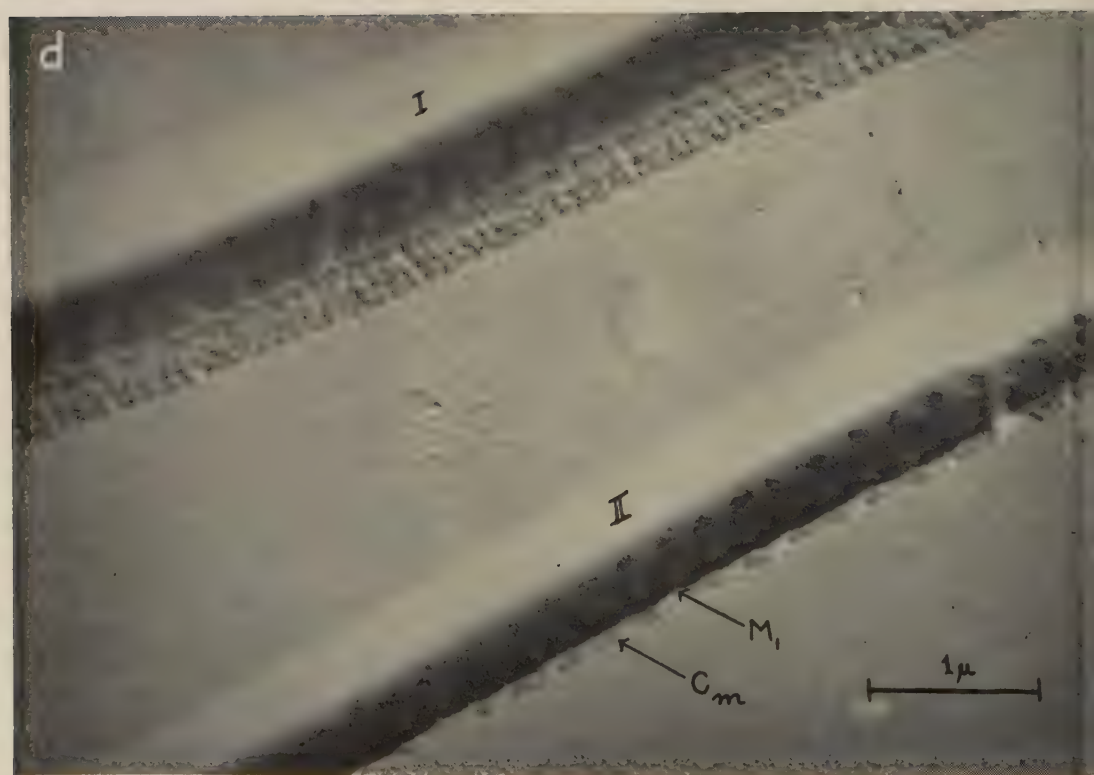
c
1 μ

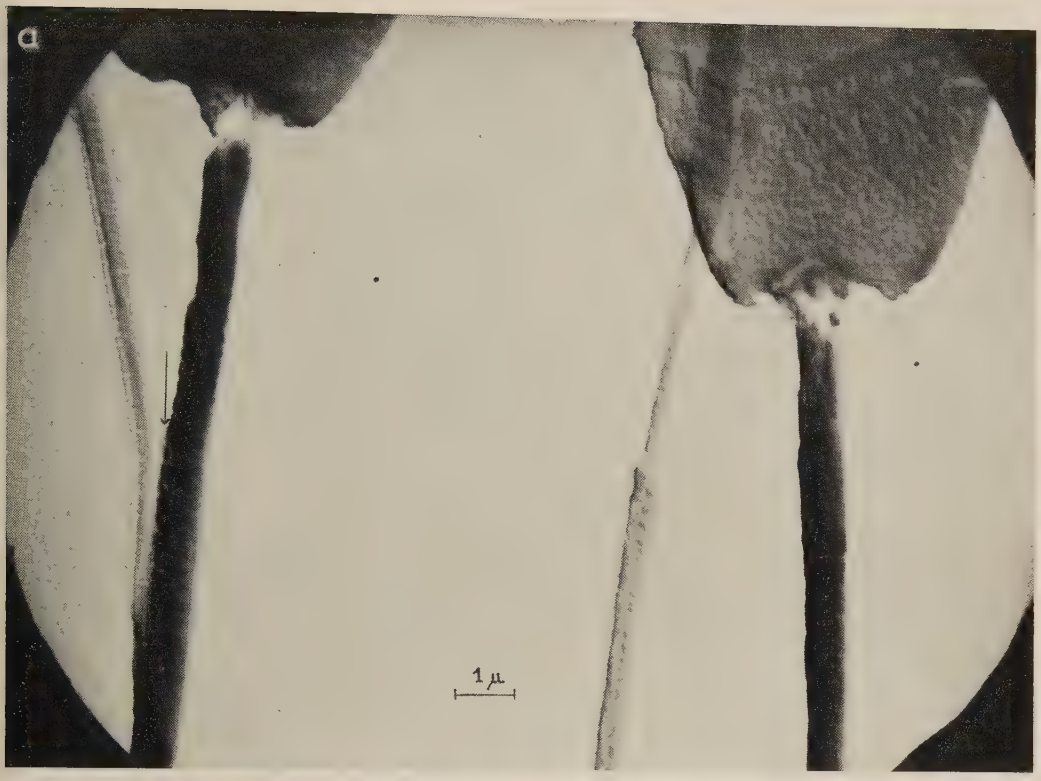

d
1 μ

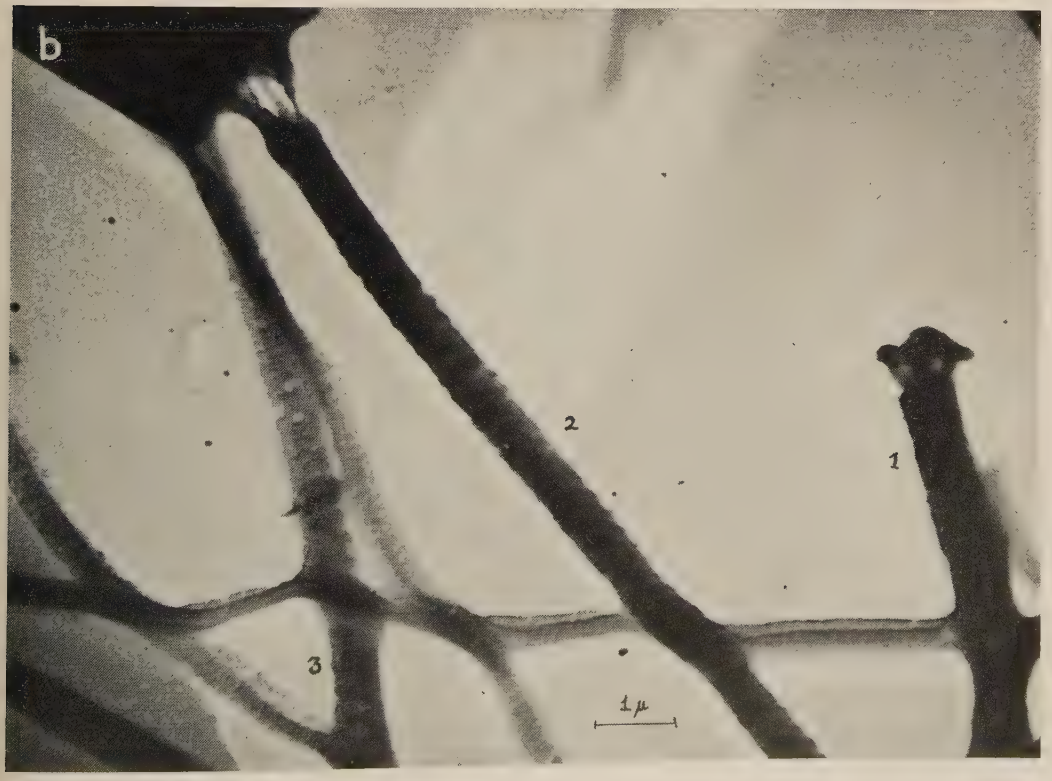
a
J
J
1μ

b
1μ

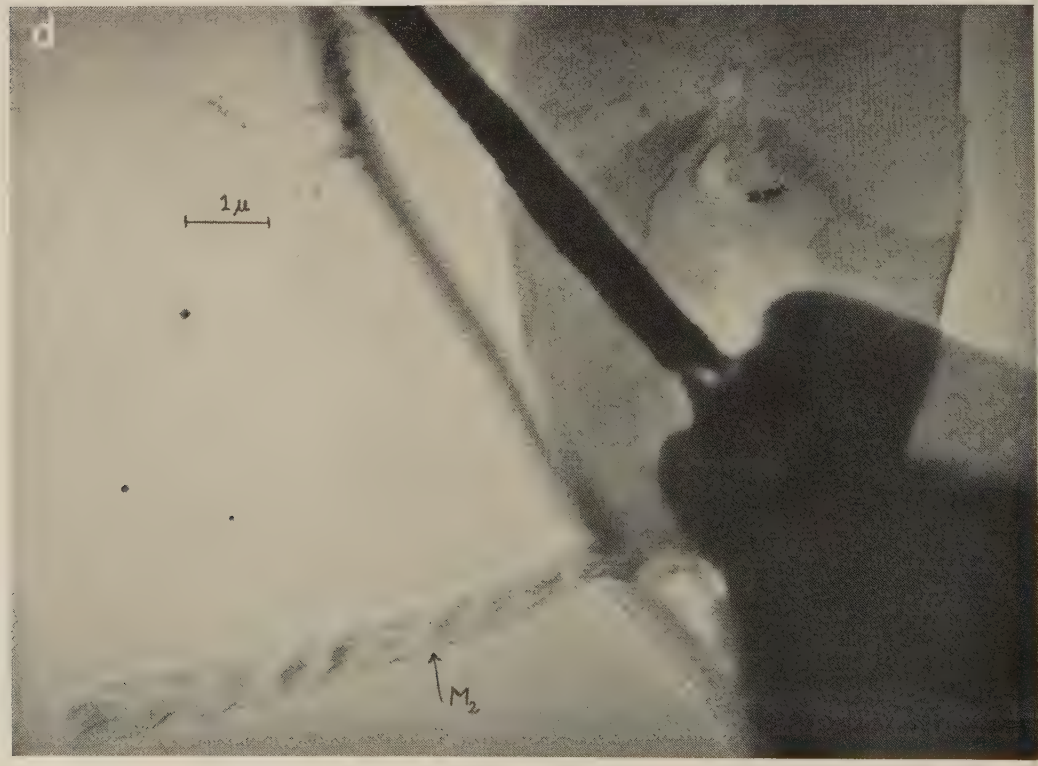

c
1μ

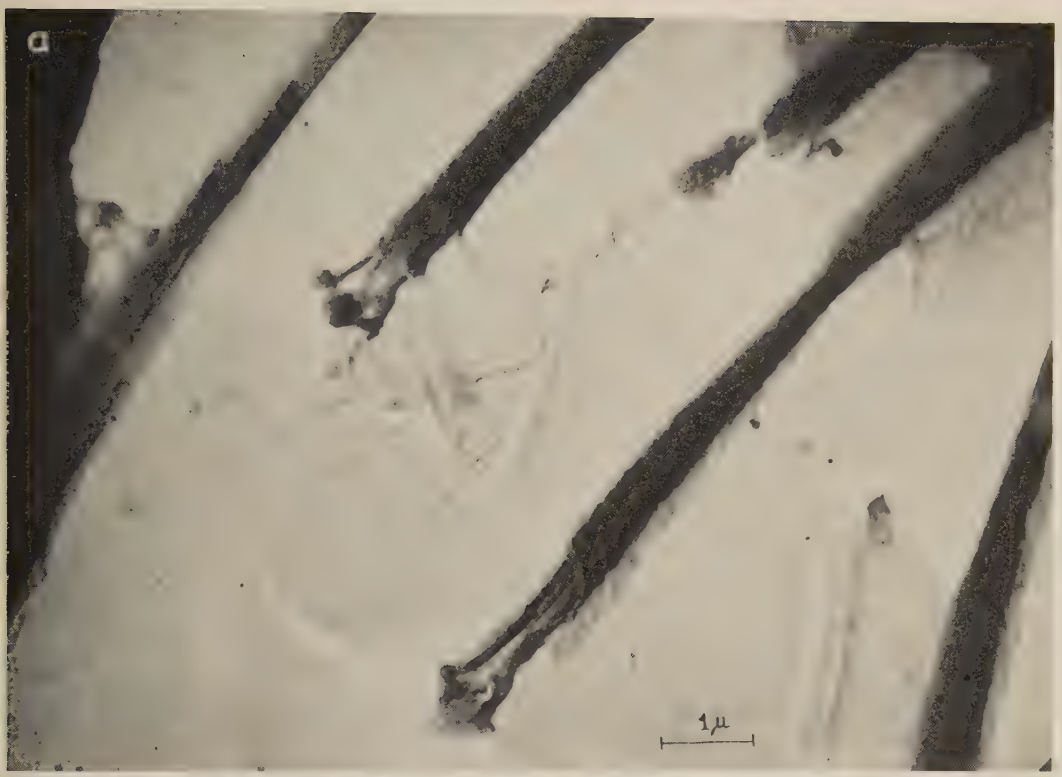

d
I
II
M1
Cm
1μ


a
1 μ

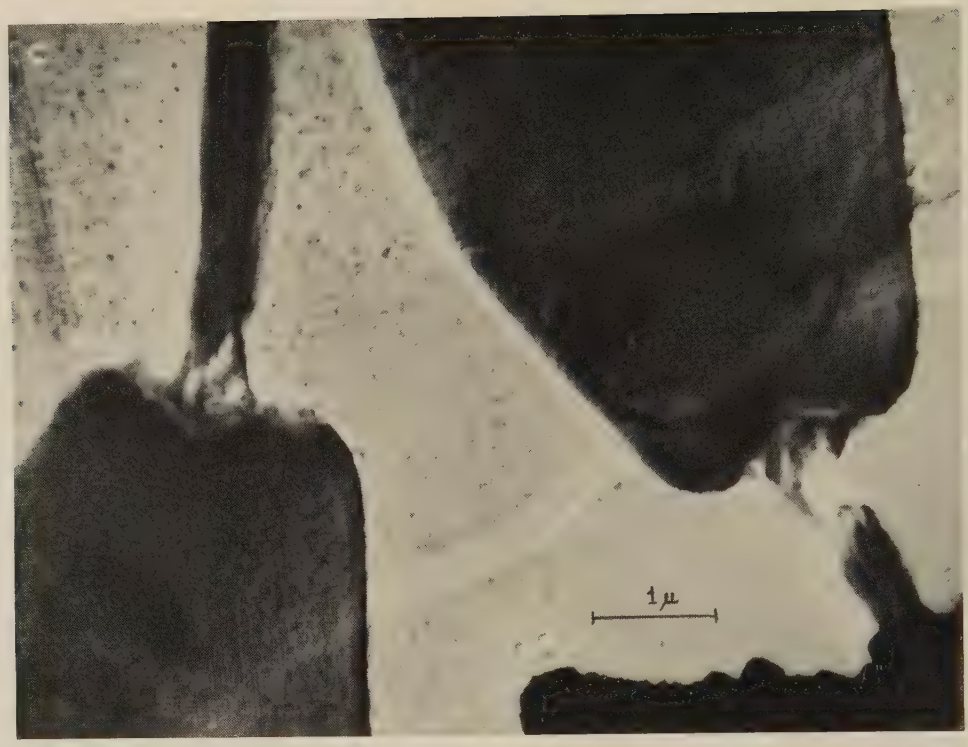

b
1
2
3
1 μ

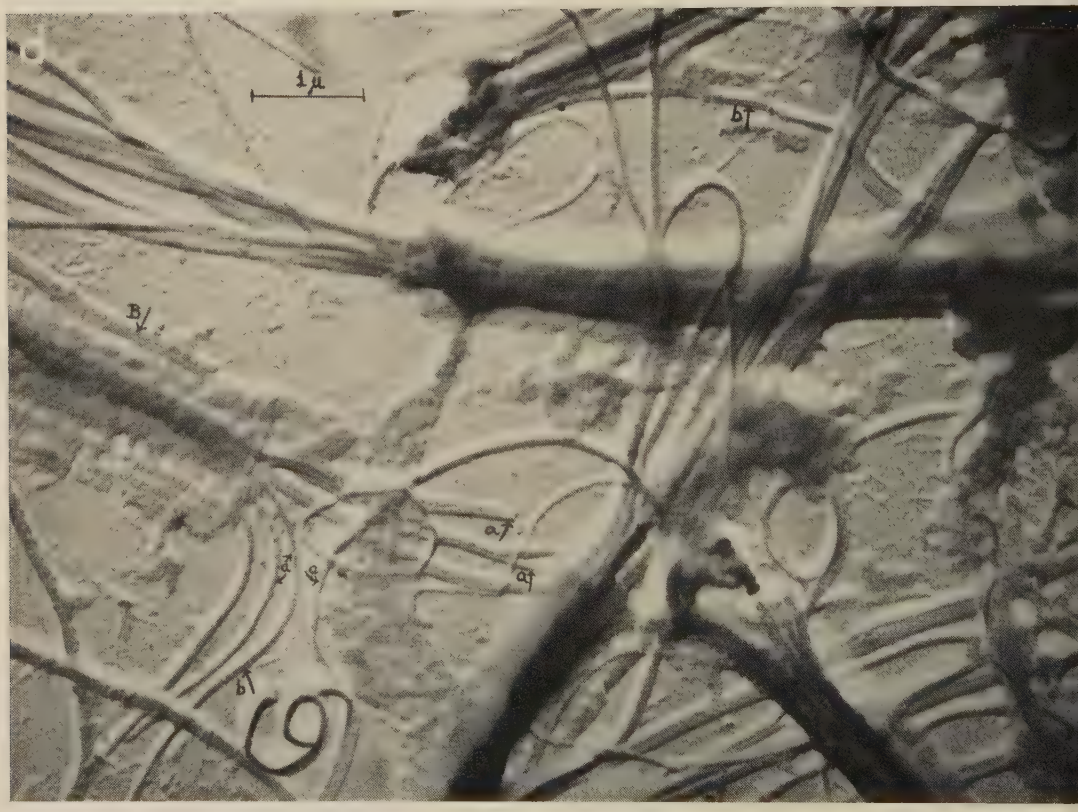
c
B
A
1 μ


d
1 μ
M2


a
1μ

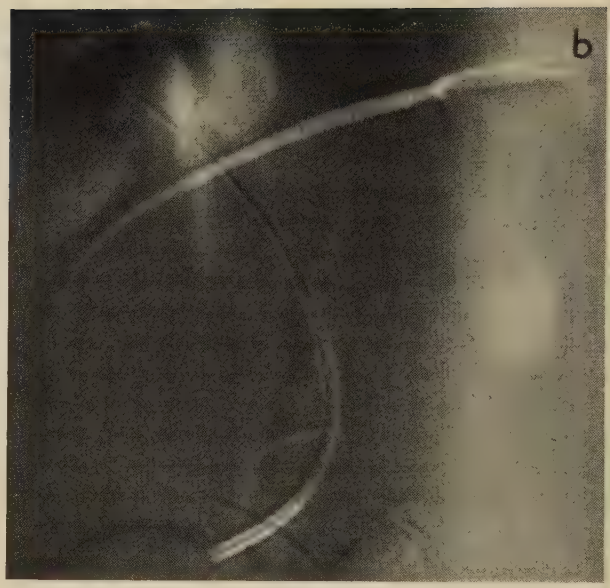
b
1μ


1 μ


1 μ
b1
B1
a1
a1
a1
b1

a
A
B
1μ
1μ
b
1μ
c
1μ
d
1μ

a


b

c

d
C
A
B

THE QUANTITATIVE DETERMINATION OF HISTOCHEMICAL REACTIONS FOR ENZYMES[1]

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Methods for the quantitation of tissue components such as potassium (6), nucleic acids (1, 2) and glycogen (4) in tissue sections by means of colorimetry (visible or ultraviolet) through the microscope have been described. In the case of enzymatic reactions, the intensity of the reaction is usually graded by arbitrary plus marks. Such grading may be highly misleading because an extensive reaction, causing an overall darkening of the section, is likely to be graded as intense whereas another tissue containing much more enzyme per gram of tissue but distributed unevenly, in minute, highly active, discontinuous areas may give the impression of lower enzymatic activity. In addition, once a black spot is obtained, activity beyond this level cannot be estimated on the basis of shade.

The following method, based on the idea of Coujard (3), permits approximate quantitation (within the limits of about ± 50 per cent) of histochemical reactions for enzymes.

A highly active enzyme solution is made according to one of the accepted methods, and its activity is assayed. Serial dilutions by a factor of 2 are made with a suitable diluent such as a 1 per cent gelatin or gum acacia solution. Standard slides are prepared as follows: carefully cleaned microscopic slides are coated thinly with egg white-glycerol, just as for histologic purposes. They are subsequently heated over a Bunsen flame until completely dry. This pretreatment will prevent the running of ink when the marks are made. With a clean steel pen parks are made on the slides, the serial dilutions being used as ink. To avoid confusion, it is best to write with each dilution its strength as a mark. The slides are dried, fixed briefly in alcohol or acetone,

[1] This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the United States Public Health Service.

coated with thin (about 0.1 per cent) collodion, washed and incubated in the substrate solution as regular histologic slides would be. Further processing of the slides is the same as that of tissue sections.

In the finished slides the marks will show up in gradations from colorless to black (or dark brown, depending on the technique; Fig. 1).

With this method it was found that a slight but distinct grey shade is produced with about 20 to 25 μM-units/ml (5) of alkaline phosphatase activity, and a definitely black shade with about 200 μM-units, the length of incubation being 1 hour. The intensity of the reaction was found to be proportional between 5 minutes and 24 hours, i.e., blackening was obtained after 5 minutes with an activity of 3,000 μM or over, and after 24 hours with an

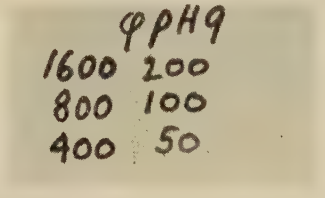

Fig. 1. A typical standard slide, natural size. Activity in μM-units. Incubation 1 hour at 37° C.

activity of about 8 to 10 μM or over. With acid phosphatase the results were similar although often irregular; with hepatic esterase the first traces of a brown shade were obtained with an activity of about 2,000 to 3,000 μM in one hour.

The approximate estimation of enzymatic activity is done by incubating a number of tissue slides together with standard slides in the same Coplin jar. At intervals (e.g., 5, 10, 20, 40 minutes, etc.) one tissue slide with a corresponding standard are removed from the incubating mixture, and the color is developed. The pair of slides in which the tissue structure in question first shows up in the shade chosen (preferably black) are used for comparison. The mark made with the lowest dilution and showing in black will have approximately the same activity per unit area as the histologic structure in question.

The specific enzymatic activity (activity/ml of tissue) of the structure can be calculated from the following data: thickness of the section, activity of the dilution, and the amount of fluid deposited per unit area of the mark. The first two data are directly available; the third one was determined in the following way: with a drafting pen a series of lines (8 to 10), 20 mm long

each and of uniform width, were drawn on small slips of previously weighed thin drafting paper. By gradually releasing the screw of the pen, the width of the lines was made to increase from 215 to 800 micra, as determined with the aid of an ocular micrometer. A 2 per cent solution of gelatin in 50 per cent glycerol, with some lamp black added, was used as ink. This ink had a density of 1.135; it flowed freely and dried very slowly. The slips of paper were reweighed on a torsion balance immediately after the lines were drawn, and the weight of the liquid deposited was calculated from the difference. For each width of lines 3 slips of paper, containing 8 to 10 lines each, were used, and the values were averaged. From the weight of the lines the thickness of the liquid layer was calculated (Table I). The latter is proportional to the amount of fluid and to activity per unit area.

TABLE I

Thickness of fluid layer in drawn lines.

A Width of lines mm	*B* Total length of lines mm	*C* Weight of lines mg	*D* Thickness of lines (mm) $\left(\frac{C}{AxBxl \cdot 135}\right)$
0.214	250	2.0	0.033
0.358	250	3.9	0.0385
0.500	250	6.9	0.048
0.573	250	8.9	0.055
0.660	250	12.6	0.067
0.740	200	13.0	0 0785
0.800	200	18.16	0.102

Since the colorimetric density of a homogeneous layer depends on the absolute amount of colored material per unit of projection area, the specific activity of any tissue structure will be identical with that of the mark showing up in the same shade, multiplied by H/h, where H stands for the thickness of the fluid layer of the mark and h for the thickness of the section, provided the structure occupies the entire thickness of the section. The value for H is obtained from the table, after measuring the width of the mark.

The inaccuracy of this method is clearly realized. First of all, it is impossible to draw lines or to make marks of absolutely uniform width; second, the profile of the cross section of a wet mark is not a rectangle but an area bounded by a curved line, not of the same shape on paper and glass. Also, the activity of the tissue determined by this method does not refer to

the activity of the fresh tissue but to the fraction of enzyme surviving the procedure of fixation and embedding. With these limitations, however, the method can be used to estimate at least the order of magnitude of enzymatic activity in a tissue detail appearing in a uniform shade under the microscope. Furthermore, the method can be used for the study of the effects of various histologic procedures such as fixation or embedding on enzymatic activity since the standard slides can be exposed to a variety of physical and chemical influences.

With some highly active tissue structures it may happen that an intense black shade is obtained within an incubation time of 5 minutes. In such cases activity can be subdued by keeping the temperature during incubation as low as 0° C. Also, suitable chemical inhibitors may be used.

So far, most of the quantitative work has been limited to alkaline phosphatase. Some of the results obtained are as follows:

1. The activity of the brush border of the mouse kidney is over 10,000 μM-units (about 300 Bodansky units) per ml of tissue. That of the cuticular border of the mouse and dog intestine may exceed 20,000 μM-units.

2. Fixation for 24 hours in 96 per cent alcohol or in acetone at ice box temperature does not cause any appreciable loss of activity.

3. Loss in enzymatic activity due to exposure to xylene at a temperature of 56° C for 1 hour is distinctly less than 50 per cent.

4. Loss in enzymatic activity of hepatic esterase, due to hot xylene under the same conditions, is between 50 and 75 per cent.

Further studies on the effect of various physical and chemical influences on hydrolytic enzymes are in progress.

Summary:— A method for the quantitation of histochemical reactions for enzymes is described. It is based on the use of standard slides on which marks are drawn with enzyme dilutions of known activity.

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THE PHYSICAL PROPERTIES OF CYTOPLASM

A STUDY BY MEANS OF THE MAGNETIC PARTICLE METHOD

Part I. Experimental

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A. INTRODUCTION

THIS PAPER has two aims: first to describe our experimental work on the physical properties of the cytoplasm of chick fibroblasts in tissue culture, with a short discussion of its implications and secondly to present such details of the magnetic particle method as to enable other workers to use it.

A second part will be devoted to the theory underlying these methods.

B. TECHNIQUE

In this section we shall describe in outline the methods we have used, leaving the details to later sections.

Our basic technique, which was suggested to us by Dr. Honor B. Fell, is to allow cells growing in tissue culture to phagocytose small magnetic particles, and then to move these particles in the cytoplasm by means of external magnetic fields. It is possible to make certain limited deductions about the physical properties of the cytoplasm from the character and magnitude of the movements.

The magnetic materials we have used have been in a finely divided form. The material is added to the culture medium of the original explant. The cultures are then allowed to grow for three days after which they are subcultivated into the normal medium. In the subsequent outgrowth, cells containing phagocytosed particles can be observed under high power. The explants were usually from the frontal bone of an 11 or 12 day chick embryo.

In applying the magnetic field to the particles within the cells we have experimented with three types of movement, which can be described as

(a) twisting, (b) dragging, and (c) prodding. We shall amplify these in turn. Our work on the two last has been preliminary only.

(A) TWISTING

We have done this in two ways; firstly, by applying a large field parallel to the length of the particle, and then turning this field through a small angle; secondly, by making the particles within the cells into little permanent magnets (by magnetising them in an initial very large field, applied momentarily) and then observing their motion when a relatively small field is applied, usually perpendicular to their length. This latter method has proved to be the more useful, and has been adopted for the greater part of our work.

(B) DRAGGING

A uniform magnetic field produces a pure twist on a magnet. In order to drag it from one place to another a non-uniform field must be used, and to produce appreciable movement, a very high field-gradient is necessary. This implies that the poles of the external magnet must be as near the magnetic particle as possible. To do this so that we can observe the particles during the movement, Dr. H. B. Fell was able to produce tissue cultures on cover-slips no more than 4 mm. in diameter. The polepieces can then be brought up to one side of this culture, and the cells can then be observed with a $\times 20$ objective.

(C) PRODDING

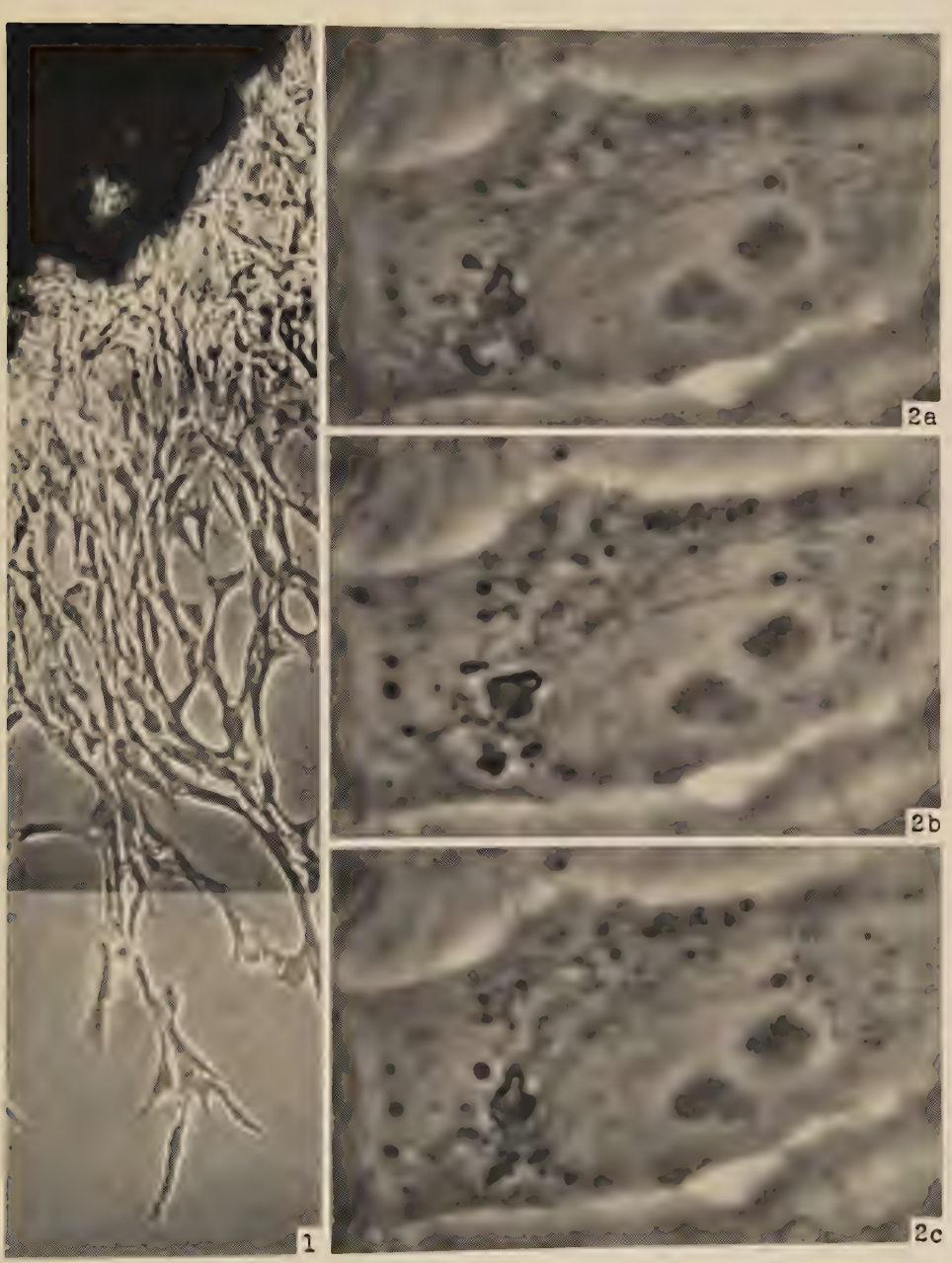
This is really a special case of the first form of twisting. If a cell contains numerous magnetic particles, a strong magnetic field will loosely unite the particles into a rod. This rod, which may be longer than the width of the cell, can be rotated by the field to bear upon the structures of the cell. We have thus been able to prod the cell membrane outwards, and the nuclear membrane inwards.

The descriptive part of the paper is presented under these three headings. However the greater part of it falls under Twisting, so this has been subdivided into a section on the details of the method and one describing most of our experimental results.

PLATE I

Fig. 1. Part of a culture of embryonic chick bone containing magnetic material to show normal outgrowth. Phase contrast $\times 200$. The magnification refers to the *unreduced* plate.

Fig. 2. One cell in the outgrowth shown in Fig. 1 containing a magnetic particle. Phase contrast $\times$ 4,900. (a) before actuation by magnet; (b) after magnet was moved across culture; (c) magnet again moved but in reverse direction. In this cell, the particle is unusually close to the nucleus). The magnifications refers to the *unreduced* plate.


1
2a
2b
2c

C. SUMMARY OF THEORETICAL CONCLUSIONS

(To be given in full in Part II)

(1) On the scale of our experiments, inertia delays the attainment of equilibrium by only a very short time (less than 1 milli-second) and can therefore be entirely neglected. This is because the particles are so small.

(2) Consider a particle within a fluid, the whole enclosed by a boundary as in Text Fig. 1, then if the scale of the system is varied, and the relative proportion of the particle and boundary is kept constant, the effect of size is as follows:

Fig. 1. Diagram to illustrate effects of boundaries.

(a) For twisting: none. The angular velocity for given magnetic conditions is independent of size. This is true for both newtonian and non-newtonian liquids.[1]

(b) For dragging: the velocity decreases as the size decreases. For a newtonian liquid the velocity is proportioned to the square of the linear dimension.

(3) The effect of boundaries at a short distance from a short particle (say at a distance $\frac{1}{4}$ the greatest dimension of the particle) is to produce an increased resistance. We consider a newtonian liquid.

(a) For twisting: the resistance is increased moderately. (Probably by less than a factor of 1.6 for the case quoted). This remains true unless the boundaries are put very close to the *ends* of the particle.

(b) For dragging: the resistance is increased much more. For the particle moving inside a cylinder, parallel to the axis, with a clearance equal to the average dimension of the particle, it is probably increased by a factor of about 2 or 3.

(4) The forces vary in a complicated way with the *shape* of a particle, but approximate methods are given in Part II for estimating them for newtonian liquids.

[1] A liquid is said to be newtonian when its viscosity is independent of shear-stress. A simple account of viscosity, thixotropy. etc. is given in Heilbrunn. 1943, page 61.

In our experiments there is also a variation due to the magnetic properties of the particles. The combined effect of these two factors is to make absolute measurements on the twisting of the individual particles only approximate (see page 55).

(5) For a non-newtonian liquid it is not possible to obtain any but the crudest estimates of the variation of viscosity with shear stress with these methods. The values given by the newtonian formulae are some indeterminate average values.

(6) The behaviour of the particles in an elastic medium can be calculated for small strains from the corresponding formulae for a viscous medium. In a visco-elastic medium it is possible to calculate the "relaxation time" (if this is constant with stress) without knowing the precise nature of the particle and the boundaries.[1]

(7) In dragging experiments, the force on a magnetically saturated particle is proportional to the field gradient. A particle is not necessarily pulled parallel to its length. A magnetically saturated particle can never be in true stable equilibrium under magnetic forces alone.

(8) To obtain a large drag on a particle, it is essential to have iron not far from the particle. The value of the field-gradient is of the order of $(B-H)/Xo$ in well-designed systems, where $(B-H)$ refers to the iron near the particle, and Xo is the distance of this iron from the particle.

(9) Air-cored coils cannot compete with magnets for producing sustained field-gradients, because of the heating effects.

(10) On *small* particles the shear stresses in the fluid due to twisting magnetically are, in general, rather bigger than those due to dragging magnetically. Both these stresses are much greater than those caused by gravity. The shear stresses produced by centrifuging natural inclusions of radius 1 μ can only equal those produced by twisting magnetically if the centrifugal fields are of the order of 100,000 g. This is in the region of the fields given by the ultracentrifuge.

D. TWISTING: APPARATUS, TECHNIQUE AND CALIBRATION

1. *Biological Technique*

The explants were obtained, unless otherwise stated, from the frontal bone of 11 or 12 day chick embryos. They were grown in hanging drop pre-

[1] The terms in this paragraph are explained in the Appendix, page 80.

parations on $1\frac{1}{4}''$ square No. 2 coverslips over $3'' \times 1\frac{1}{2}''$ hollowground slides. The medium consisted of equal parts of chick embryo extract and fowl plasma. When required a small amount of a dense suspension of the magnetic material was added to the fowl plasma. In the early experiments it was sometimes impossible to see the explant clearly because of the black magnetic material.

After incubation, usually for three days, each culture was transplanted, generally into two fresh hanging drop preparations. No more magnetic material was added to the new embryo extract — plasma medium. The outgrowth was cut away except for a narrow margin round the original explant. This time a No. 1 coverslip was used, which was mounted on a special metal ring, with another coverslip on the lower side of the ring, to facilitate observation. Both were sealed on with paraffin wax (Hughes and Fell, 1949).

The explants still contained magnetic material, and many of the fibroblasts growing out into the medium contained one or more magnetic particles which they had phagocytosed. The growth appeared normal and vigorous, and it was possible to sub-cultivate several times satisfactorily. On each occasion some of the cells migrating into the new medium contained magnetic particles.

This particular method of preparation and mounting allows easy observation of individual magnetic particles in cells migrating into the clean medium.

The special cultures developed for the dragging experiments are described on page 70.

2. *Possible Biological Artefacts*

One of the advantages of the method is the comparatively small disturbance of the cell which results because the particles enter the cell 'naturally' without forcible introduction as in the experiments of Seifriz (1924). We have not made quantitative studies of the rates of growth of cultures with and without the magnetic material, but we can state that there is no obvious difference. Cultures with a layer of magnetic material so dense that it was difficult to see the explant have been known to grow as actively as control cultures. In such extreme cases it is difficult to place the knife cuts when subculturing, and this would account for certain cases where the growth was poor on one side. However, in the cultures with smaller amounts of magnetic material this caused no difficulty, and the rate and amount of growth appeared very similar in both treated and control cultures. Moreover, the appearance of the cells — the amount of mitochondria, fat, vacuoles and so forth — though naturally very variable, showed no consistent difference due

to the particles, nor in a given culture did cells without particles appear obviously different from cells with them. Cells containing much magnetic material passed normally through mitosis.

Although in general we have only used the first subcultures on one or two occasions we have for special purposes subcultivated several times, when the growth was always normal. It is thus fairly certain that the magnetic material produces no gross interference.

It is possible that the particle inside the cell is within a cytoplasmic vacuole. If this were so, our twisting experiments would measure some property of the vacuole rather than of the cytoplasm itself. Such vacuoles, which show clearly in the phase contrast microscope, can accumulate neutral red, and show active Brownian motion in older cultures during the second day after sub-cultivation. Small magnetic particles appear to become attached to the surface of these vacuoles. (If they were free inside they would quickly fall to the bottom.) It is not easy to see whether they are just inside the surface, or just outside, because one is working near the limits of resolution of the optical system.

Particles attached to vacuoles behave in a characteristic way when a magnetic field is applied. Firstly they show translation in addition to the usual rotation. This is presumably because they are constrained to move only in the surface of the vacuole. Secondly, the movement is typically very irregular and abrupt. For example, they may not move at all when the field is switched on, and then suddenly move rapidly to another position. We have not studied this movement in any detail.

Although we can be sure that a magnetic particle apparently free in the cytoplasm is not attached to a large vacuole, it is not absolutely certain that we could detect optically a vacuole which just surrounded a medium-sized particle. The vacuoles, however, are typically spherical and we could probably detect a vacuole around an elongated particle. Moreover, we might well expect the vacuole to grow with time, so that even if we could not see it at the beginning of an experiment it would be visible several hours later. This we have never observed.

In two cases after measurements had been completed on a particle we stained the living culture with neutral red. It is not certain that this method would disclose a tightly-fitting vacuole any better than phase-contrast, and there is the additional danger that the neutral red itself may produce a vacuole. However, in these instances no enveloping vacuole was apparent, although in one cell a very small watery vacuole was demonstrated near the fairly large particle, as had been suspected from the phase-contrast observations.

We think it improbable, therefore, that the particles we have selected for measurement were actually in watery vacuoles. In our later experiments we were careful to select cultures and cells which as far as possible were free from vacuoles. We have found, incidentally, that cultures from the same batch may vary considerably in degree of vacuolation of their cells.

There remains, however the more profound objection that the particle, while not producing a vacuole, may modify the physical properties of the adjacent cytoplasm. This is clearly impossible for us to refute. The only approach would be to repeat the measurements by an entirely different method, and this we have not been able to do.

It may be worth while to note that certain modifications might only have a small effect on the rate of twisting. For example, if the cytoplasm solidified (or liquified) around the waist of an elongated particle, but not at its ends, this would have little effect, as the greater part of the viscous couple comes from the forces at the ends of the particle. This is not, of course, a very likely alteration, although a small vacuole might conceivably form there. Again, if the protoplasm formed a solid layer a fraction of a micron thick all round the particle, this would only increase the viscous couple slightly, as the shearing would simply take place a little further out.

3. *Magnetic Materials*

We have regularly used two magnetic materials, and tried two others. The first was magnetite, an oxide of iron, prepared in colloidal form. It has ferromagnetic properties, but much less than those of iron or steel. The material we have used most was originally prepared as finely divided iron, but by the time we came to use it was largely oxidised. However, it had a much greater retentivity than the magnetite, and was thus more suitable for our second method of twisting.

The other magnetic materials we have tried were finely divided nickel, which proved to be toxic to the cultures, and powdered 'Alcomax', which proved variable in its magnetic properties, so that no further work was done on it. We shall only describe here the experiments with the first two materials.

MAGNETITE (Fe_3O_4)

This was prepared by Lefort's method, given by Welo and Baudish (1927). That is:

"281 grams of $Fe_2SO_4 + 10H_2O$ and 134 grams of $FeSO_4 + 7H_2O$ are separately dissolved in 750 cc. and 500 cc. of water, respectively. They are then mixed, and poured into a boiling solution of NaOH containing 250 grams of the hydroxide in 3,200 cc. of water. The black mixture formed on the addition of the sulphates is boiled for an hour. The mixture is near colloidal, so that the precipitate does not settle readily."

It was then washed with distilled water and centrifuged, repeatedly until neutral to litmus paper, after which it was dried in a desiccator.

We have not determined the B/H curve for this material, as we only used it for our preliminary experiments; where necessary we have used the one given by Welo and Baudish. It is possible that the ordinate of their curve should be multiplied by a factor to allow for the looseness of packing.

OXIDISED IRON

This was supplied to us by Dr. K. Hoselitz, Director of Research of the Permanent Magnet Association, for whose assistance and advice we are very grateful. He has sent us the following account of the method of preparation:

"We made an alloy of 50 per cent by weight of aluminium in iron in the high frequency furnace. This alloy, which is hard and brittle, was easily crushed and ground in a planetary ball mill. After grinding, the powder was graded and 100 gm of the following grading was selected:

$\frac{1}{2}$ passing 200 mesh.
$\frac{1}{6}$ passing 160 mesh, resting on 200 mesh.
$\frac{1}{6}$ passing 90 mesh, resting on 100 mesh.
$\frac{1}{6}$ passing 30 mesh, resting on 60 mesh.

This sample was well mixed, and then slowly added to an NaOH solution consisting of 100 gm of caustic soda in 400 gm water. This caused the aluminium to be dissolved out of the alloy. The reaction is exothermic and the solution soon reaches the boiling point, which accelerates the reaction and no attempt at cooling is made.

After the reaction has ceased the solution is filtered through a Buchner Funnel with 540 filter paper and washed with clean water until the washing water is no longer caustic. The wet sludge retained on the filter is scraped into a bottle and covered with distilled water for storage."

We kept the material under water and when a sample was required, the bottle was shaken and allowed to stand for a couple of minutes to permit the coarser material to sink. The top layers were pipetted off, and the iron was washed in glass distilled water and centrifuged repeatedly. It was then dried in a desiccator and used as required. Dr. Hoselitz has pointed out to

Fig. 2

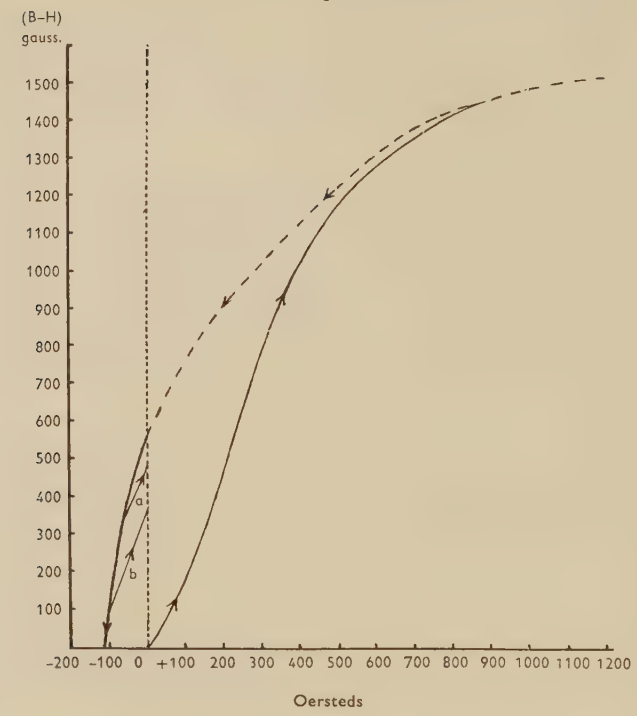


Fig. 2. (B–H) Curve of oxidised iron. (a) and (b) are recoil curves.

us that if the powder is dried it oxidises visibly, so that as we used it the material was substantially oxidised iron. An analysis by Messrs. Geochemical Laboratories has confirmed this. The percentage composition is as follows:

Iron	61.0
Aluminium	5.4
Combined oxygen	28.3
Water	2.1
Silica, manganese	2.2

The B/H curve was found by pressing the powdered material into a glass tube so that it formed a cylinder 15 cm. long and 0.4 cm. diameter. The length/diameter ratio was therefore so great that demagnetising effects could be neglected. The method used was the ballistic one, a flux coil being wound on the glass tube, and the whole placed inside a long solenoid. The curve of (B-H) v H is given in Figure 2. The values have been corrected for the effect of packing, by increasing the (B-H) figures in the ratio of

$$\frac{\text{apparent density}}{\text{true density}} = \frac{4.2}{2.0} = 2.1$$

so that they should represent average values for individual particles.

Owing to the low permeability the results are not very accurate, but they are good enough to show the essential features of the curve. The permeability is low (maximum about 3.6), and the material saturates at (B-H) equal to about 1,500. The back part of the curve shows that the effective value of (B-H) for ellipsoids of different length/diameter ratios does change, but not enormously.

TABLE I

Length/diameter ratio	1.0	2.0	3.0	5.0	∞
Effective (B-H)	225	325	390	460	550

The effective value of the slope of the curve over the part which interests us, is about $4\frac{1}{2}$. The recoil curves show that a field of 50 oersteds applied in reverse momentarily to a short particle (axial ratio 2 : 1) may permanently lower its magnetic moment, probably by about 20 per cent. It would therefore be inadvisable to use fields much greater than this. Fields of this magnitude would not give exactly the usual variation of couple as cos θ (where θ is the angle between the applied field and the magnetic axis of the particle), but for our purposes the differences are not important.

4. *Methods of producing magnetic fields*

Our first method consisted in mounting a large U-shaped Alcomax magnet horizontally with the objective in between the poles. The size of the faces was 1.9×2.2 cms and the distance between them 2.2 cm. The mean length of the U was 13 cm. The bottom edge of the magnet was a few millimeters above the level of the culture. This magnet could slide horizontally in and out, so that the strength of the field could be varied from about 800 oersteds to 20 oersteds. It could also be rotated about the objective to change the direction of the field, over an arc of about 150°.

This magnet was used for the original experiments with magnetite. By simple mechanical means the magnet was made to perform oscillations of $\pm 15°$, with periods of the order of 5 to 15 seconds. This was done by means of an electric motor, a reduction gear, and a series of levers. This magnet was also used for the experiments on prodding, where a large field was re-

quired. In this case a different mounting was used which enabled the magnet to move round the full 360°.

Such a method suffers from the disadvantage that the magnetic field cannot be turned on and off either conveniently or quickly, so for most of our experiments we have used fields produced electrically. The main field was produced by a horizontal electromagnet. This consisted of a μ-metal rod, diameter 1.4 cm, length 14 cm, wound with about 900 turns of wire. This was mounted with one end of the rod 2.75 cm from the axis of the objective, and the end of the microscope stage was cut away to give necessary clearance. This electromagnet proved very satisfactory. The magnetic field was linear with the current up to about 50 oersteds, after which the metal saturated rapidly. The maximum field was 65 oersteds. There was no detectable hysteresis, as might be expected. In the experiments an air-cored coil was used to produce a small horizontal field at right angles to the first. This was mounted on the back of the hot-box about 8 cm from the objective. It gave a field of only 10 oersteds for a current of 4 amps, and it was thus not really strong enough. In our later experiments, therefore, we have used a second horizontal electromagnet mounted on the stage of the microscope. This has the same kind of μ-metal core as the first one. At a point near the centre of the magnet, but 3 cm from its axis, it gives a field of 60 oersteds for 1.2 amps, after which it saturates very rapidly. As now mounted, the second electromagnet moves with the mechanical stage, so that the field it produces under the objective for a given current varies a little. However, as it is only required for moving the particles into position or waggling them violently, this does not matter. Exact measurements have always been made when the first electromagnet was used. To produce vertical fields a small air-cored coil in the shape of a ring was mounted on the substage condenser, with its axis in line with that of the optical system. This had a mean radius of 3.6 cm, and was wound with 75 turns of 30 S.W.G. wire. This again was not fixed in position, and it was only used for manipulation. It gave a field of about 20 oersteds for 1 amp.

The calibration of these fields was done with a search coil and a galvanometer. The second electromagnet, as might be expected, slightly increased the field due to the first.

The heating effects for the kinds of currents used in the various coils was negligible except for the coil mounted on the sub-stage condenser. This produced a rise of $\sim\frac{1}{2}^\circ$ C in the culture for a current of 2 amps for 1 minute. It was thus necessary to use this coil for short periods only, but in practice this has not proved a disadvantage.

5. *Residual Fields*

These fall into two classes: permanent fields, like that of the earth which can be compensated for; and varying fields, which alter as the different fields are turned on and off in the various coils, and which are due to the effects of iron, or other ferromagnetic material in or near the objective.

Early in the work it was found necessary to remove the steel springs and a steel rod from the movement of the microscope stage. As our experiments became more precise we were compelled to replace the steel ring in the substage condenser by a brass one. This proved fairly satisfactory until we began to use the phase condenser and objective. Both were appreciably magnetic. The major part of the effect due to the condenser was, rather surprisingly, removed by taking off electrolytically the plating on the brass ring which fits into the condenser holder. The adjusting rods were replaced by brass ones. The condenser still has magnetic material in the centre of the large phase ring, but this is far enough away for its effects to be very small.

The magnetic effect of the objective (due to the plating?) is quite appreciable, and we have temporarily overcome this in our most recent experiments by changing the objective to an ordinary one which is non-magnetic when we wish to make or record exact measurements. This is not as great a disadvantage as it might seem because the actual particle is more easily visible without phase-contrast than with it.

Unfortunately we had recorded some of our results before the magnitude of the residual field from the phase-contrast set-up was realised. We have not discarded these results, but as will be explained later, certain features of the curves must be regarded as possible artifacts.

The fixed fields are more easy to compensate. A ring-shaped coil, mean diameter about 14 cm, mounted on top of the hot-box with its axis coinciding with the optical axis, compensates for the vertical residual field. It has 30 turns of 24 S.W.G. and its usual current is about 0.1 amps. The remaining horizontal fields are compensated by a bar magnet (two at right angles would be more convenient) outside the hot-box. Naturally this must be done with the μ-metal electromagnets in position. The vertical compensating coil must also be roughly adjusted, as it produces a horizontal component due to its action on the μ-metal rods.

It is interesting to note that the large iron feet of the microscope, the top of the hot-box heater, and the tall steel pillar on which the camera is mounted,

produce a negligible effect on the *variable* part of the residual field. It is clearly only magnetic material which is both near one of the field-producing coils and not too far from the objective which causes trouble.

The actual adjustment was done by making up a slide of some of the magnetic material in a mixture of 90 per cent glycerine and 10 per cent water (if at room temperatures), magnetising, and then observing the behaviour of individual particles under the microscope. The particles must of course be very widely spaced, and not near any big fragments. The residual horizontal field is then found by applying a small field due to the main electromagnet until the particle roughly bisects the angle between its position with no applied field, and its position in a very large applied field. The small applied field necessary to do this is then equal in magnitude to the residual field.

The vertical field is adjusted roughly first. After the first compensation of the horizontal field this adjustment is refined. The technique is to choose a long particle, and move it so that it is subtending the maximum length to the eye. This can only be approximate, but is quite adequate if the horizontal residual field is already very small.

Under the best circumstances the horizontal residual can be reduced to a field of about $\frac{1}{10}$ oersted, which may vary in direction as the various coils are used. In practice it is probably safe to assume that the residual is less than $\frac{1}{5}$ oersted. With the phase objective, however, a change of 0.6 oersteds can be observed after the main field has been full on in one direction, and then in the other. Before the phase condenser ring was deplated the effect must have been bigger than this.

It is not however sufficient to eliminate external magnetic fields due to the microscope, etc. Since there is magnetic material in the culture itself it is important to know how big an effect this can produce. Before undertaking exact measurements, therefore, we made preliminary experiments to estimate this. At the time it was usual to use a large amount of magnetic material in the explant. Two cultures were chosen, one with such a large amount of magnetic material that the culture could hardly be seen, and the other with rather less, but still more than average. The cultures were magnetised in the usual way — that is, by a very large field perpendicular to the plane of the coverslip. They were allowed to grow, and then dried by exposure to the air. The new growth was cut away, leaving the magnetized explant in position. Glycerine, with about 10 per cent water, and containing a small number of particles of the usual magnetic material, was added. The behaviour of these particles in the fluid near the explant was then ex-

amined. As might be expected, particles far from the culture were pointing approximately vertically (as the culture had been magnetised vertically), whilst those nearer to the explant pointed in various directions. The strength of the field was estimated by the "bisection of angle" method just described though naturally the result was only approximate. There was a considerable vertical field ($1\frac{1}{2}$ oersteds) at distances of 150 μ from the edge of the explant. However, in the case of the less densely magnetised culture this residual field fell to $\frac{1}{2}$ oersted, or less at about 300 μ from the explant. We therefore decided to reduce considerably the amount of magnetic material in the culture, and to work only with particles at some distance from the explant. Moreover, under these conditions any small residual field still remaining will almost certainly be vertical, and is thus not as serious as if it were horizontal.

There remains the question of the *changes* in the residual fields produced by the culture due to the external fields used. This might be expected to be small, and with moderate amounts of magnetic material in the culture it was shown to be certainly less than that due to the remainder of the system, i.e. less than $\frac{1}{10}$ oersted.

Finally the possible effect of magnetic particles in the zone of growth must be considered. These are very sparse, and confined to a plane, so that we need regard only the nearby particles. We can estimate crudely the effect of such a particle by treating it as roughly equivalent to a sphere of diameter equal to its greatest dimension (which we will call l) since this can hardly give an underestimate.

The maximum field due to such a particle at a distance r is:

$$\frac{2\,\mathrm{M}}{r^3} = \frac{2}{r^3} \cdot \frac{(\mathrm{B\text{-}H})}{4\pi} \cdot \frac{4\pi}{3} \cdot \frac{l^3}{8}$$

$$= \frac{(\mathrm{B\text{-}H})}{12} \left(\frac{l}{r}\right)^3 \text{ oersteds.}$$

Taking (B-H) = 225 we see that for the field to be $\frac{1}{10}$ oersted or less, we must have $\frac{r}{l} > 6$. This gives a rough working rule for gauging the effect of neighbouring particles. It should be noted that these particles may be rotated by the applied fields, so that their fields may change direction in a complicated manner.

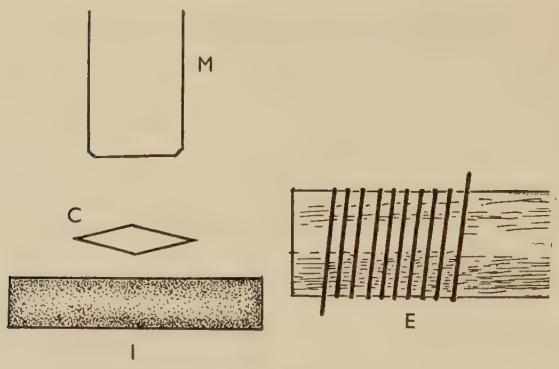


Fig. 3. Diagram to explain pseudo-elastic effect.

6. *Magnetic pseudo-elastic effects*

The residual magnetic effects discussed above are naturally rather small, and if merely regarded as a small percentage error in the main field applied they would be quite negligible, in view of the very low accuracy to which we are working. It is the fact that they persist after the main field has been switched off which makes them important, and, in particular, that their direction may be opposite to that of the main field. This can easily be seen by considering Text figure 3 which shows diagramatically an electromagnet, E, a compass needle, C, under the microscope, and a mass of iron, I. Imagine that the electromagnet produces a field with direction right to left. This will magnetise I in roughly the same direction. The compass will also point to the left. If the electromagnet is now switched off, it will produce no further effect, having no hysteresis. The iron, I, on the other hand, may possess some residual magnetism, with direction right to left. This will now deflect the compass to the *right*. It can easily be seen that the direction of the effect depends on the relative position of compass, iron, and electromagnet.

The effect on the particle in our experiments, if the direction of field is reversed, is to simulate an elastic recoil. However, even this might not be serious, as the residual fields are rather small, were there not evidence that the effect is exaggerated by the thixotropic nature of the cytoplasm. When the couple on a particle in a cell is reversed, it moves initially much more easily than at the moment before reversal. This suggests the possibility that small reverse fields might produce quite appreciable recoils. The matter is discussed more fully later. A group of particles may show a pseudo-elastic effect if they rearrange themselves, e.g. straighten out, when the field is re-

moved, and moreover the effective properties of composite particles would change with rearrangement. For these reasons, measurements have been restricted to single particles within a cell. We should perhaps mention that with single particles in castor oil we have never detected any elastic recoil.

7. *Calibration*

The magnetic properties of the particles were measured by observing their behaviour in castor oil when under the action of magnetic fields. A slide was prepared with a very small quantity of the material, which was crushed with a glass rod, and mixed with castor oil. Two no. 1 coverslips, one on either side, served as spacers, and a third coverslip was put over the top, and lightly waxed down. A $\times 40$ dry objective was used so that the lower surface of the castor oil could be observed.

The particles were carefully selected so that they were not near others, nor above a large collection of material on the bottom, nor near either the top or bottom surfaces of the preparation. They were also selected within arbitrary limits of size, namely between 2 μ and 10 μ for their greatest dimension. Only single particles were chosen, and the slide was used as soon as possible after preparation, so that few particles, except the very large ones, had time either to come together or to fall to the bottom.

To measure the angles an eyepiece with an angular graticule, produced photographically, was used and sometimes also a device for measuring the angle through which this eyepiece was turned.

In the later method in which the field was applied approximately perpendicular to the particle, the particles were magnetised before measurement, by applying a permanent magnet with conical pole pieces for a moment to the slide. This gave a field of several thousand oersteds. For timing, a small field produced by the main electromagnet was used, and the time to move through a preselected angle was measured with a stop-watch. The field was usually $2\frac{1}{4}$ oersteds in strength and the particles were timed from 45° to 135°, where 90° represents the position perpendicular to the applied field. The scatter of times was fairly great. An example of a set of 20 particles of the oxidised iron from the same batch of material gives (arranged in order): 1.6, 1.8, 2.3, 2.3, 2.4, 2.7, 2.9, 3.4, 3.7, 3.7, 3.8, 4.2, 4.3, 4.8, 5.0, 5.1, 5.5, 7.4, 9.2, 9.9 seconds. Each time is the mean of 3 readings (standard error $\sim 1/3$ seconds). This variation naturally includes variation in shape as well as in magnetic properties. The median is 3.75 seconds. As to the variation with the passage of time, the above figures were taken in July 1948, from

the material received in November 1947, which had been standing in water since then. A check in November 1948 gave a median of 4.4 seconds for a sample of 10, but at a lower temperature. Correcting for this brings the figure to 3.1 seconds, so that there was certainly no gross change in the material with time. Intermediate checks gave similar agreement. We have not considered it worth while for our purpose to press the accuracy further. The mean value of the greatest dimension of the particles used was estimated as 5 μ.

We have also made measurements on a few particles to check that the magnetic couple is roughly proportional to the field. The movement of the particles in castor oil was recorded photographically by the method used for the particles in the tissue culture (see page 55). The fields ranged from $2\frac{1}{4}$ oersteds to 36 oersteds, each being double the previous one. The slopes of the curves of angle versus time, at the 90° position, were proportional to the applied field to within ±15 per cent. The only measurement at 80 oersteds also falls within this limit. The experiments were done at an early stage before the technique was refined, and it might well be possible to improve this figure which, however, is quite satisfactory for our purpose.

The viscosity of the castor oil was checked by the falling sphere method at two temperatures. The values found, 16 poise at 14.7° C and 7 poise at 24.8° C, are reasonable.

The variation observed between the behaviour of particles in a set is bigger than would be expected if it were solely due to variation of shape. Application of the standard theory, as set out in Part II, gives the following relative angular velocities for various ovary ellipsoids.

Table II

Length/diameter ratio	1	2	3	5
Relative angular velocity	1.0	0.86	0.64	0.27

Most of our particles had length/diameter ratios of 2 or 3 to 1, and never as much as 4 to 1, so that even allowing for the simplifications of the theory we should not expect a variation of much over 2 to 1 in the angular velocity, whereas we actually get about 5 to 1. This must be due in part to the variability of the material, and a closer examination of the results supports this, since particles of apparently similar shape can vary considerably in angular velocity. The variation could be quite serious if we were concerned with obtaining accurate values rather than orders of magnitude but in the present

study we have included the shape variation with the other variants. For more precise measurements it would be better to use a more homogeneous material.

This grouping together of results is justified to some extent by the reasonable agreement between the value of the viscosity of the castor oil found by the magnetic particle method and by the normal method. If we take our average particle as having a length/diameter ratio of 2:1, and calculate the value of η by using the ellipsoid theory and the B/H curve, we obtain from the figures quoted above a value of $11\frac{1}{2}$ poise (at 19.7° C) whereas the falling sphere method gives $10\frac{1}{2}$ poise by interpolation.

8. *Photographic recording*

After our preliminary experiments it became clear that the velocity and to a certain extent the amount of movement of a particle could not be estimated sufficiently accurately by eye. We have therefore used cinematographic recording, usually at 9 frames per second, but occasionally at 15 frames per second. An 'Ensign' 16 mm camera was used, with a viewing eyepiece in a side-tube, into which part of the light beam from the microscope was deflected by a semi-reflector. This camera has previously been used to record at low magnification the beating of the heart of a chick embryo (Hughes 1949). A rough check showed that the speed of running of the camera was sufficiently constant for our purpose. The film used was usually Kodak R. 55, which is rather coarse-grained, but a more finely grained film would have needed more light, which we felt it would be better to avoid. The light was only used at full intensity for the actual time of recording apart from the necessary focussing.

During the course of the work a marker eyepiece was devised, by means of which a spot of light could be projected on to one side of the film. The time at which the circuit of the electromagnet was opened and closed could thus be recorded on the film.

The overall magnification with the 1/12th objective was about ×330. The particle was photographed using the normal illumination.

To measure the angle of displacement, the film was projected downwards onto a horizontal table with a total magnification of about 5,000. A sheet of mm graph paper was pinned to the table, and the projector adjusted so that the edges of the frames of the film were parallel to the lines of the graph paper. A small loose sheet of white paper, with a straight edge, was now placed so that the image of the particle (white on a dark background) fell

on it. Holding the paper fixed, the observer traced a faint pencil outline of the particle on the paper. The procedure was then to fit this outline to the image of the particle from each subsequent frame of the film in turn; the angle between the straight edge of the paper, and the lines on the underlying graph paper was measured with a large circular protractor. The zero of the angle was rather arbitrary, but the 90° position was chosen such that the major axis of the particle appeared to be roughly perpendicular to the magnetic field.

The largest error in this technique is the fitting of the pencil outline to the projected image. This error varies with the size and shape of the particle and the accuracy of focus. There is also occasionally the difficulty that if the particle does not always remain in the horizontal plane it may appear to change shape. Accurate measurement in such cases is clearly impossible. The root mean square deviation was usually between 1° and 2°. The angle was in general only measured once for any frame, and in passages where the particle was effectively stationary, only every fifth or tenth frame was measured.

The procedure is tedious and time-consuming, the maximum rate of measurement being about 120 readings an hour. For this paper about 8,000 frames have been measured.

The above remarks about errors apply to a slowly moving particle. There is an additional error in measuring the velocity of a swiftly-moving particle, due both to the blurring caused by movement during the exposure, and to the time lost between frames. These are best regarded as errors in the time to which the measured angle corresponds. It is difficult to estimate them accurately, but a rough guess can usually be made in any particular case.

9. *Other details*

The arrangements for controlling the temperature were quite straightforward. The hot-box consisted of a roughly rectangular wooden box, 34 × 24 × 16 cm, with the top and front of thin transparent plastic. The heater was in the metal base of the box, and was supplied from the A.C. mains. The lower half of the microscope, from a point $3\frac{1}{2}$ cm above the stage, was enclosed in the box. A subsidiary heating coil was wound at the bottom of the microscope tube, just above the objective, to prevent a large temperature gradient at the level of the culture. The heating current was controlled by a bi-metal strip mounted behind the microscope and the temperature was measured by a thermo-couple in the immersion oil.

The controls for the focusing of the objective and the movement of the stage could be operated without opening the box. The large magnet, when used, could also be moved from outside, and all the currents in the coils were similarly controlled.

The electric circuits require no special comment. They were run from two 12 volt car batteries.

E. TWISTING: EXPERIMENTS

1. *Preliminary experiments*

The first experiments were done at 37° C, magnetite particles within the cells being used. We will only sketch the details here. A large horizontal magnetic field (600 oersteds) was applied by means of the U-shaped magnet, which lined up the particles in the cells. This magnet was then oscillated mechanically in a horizontal plane through $\pm 15°$, with a period of about 12 seconds. Pictures were taken with phase contrast, at a rate of one every 3 seconds and from these the amplitude of oscillation of the chain of particles was measured. A rough calibration was made under similar conditions with castor oil. A simple theory made it possible to deduce how the amplitude of oscillation should vary with magnetic field, damping force, and the period of oscillation. If taken at its face value it gave a high figure for the viscosity of the cytoplasm, similar to that for castor oil.

These preliminary experiments suggested that the physical properties of the cytoplasm were very different from those of water. It was not possible to interpret them any more accurately because of the difficulties of calibration when long chains of particles are used, and because it is not possible to distinguish between viscous and elasticoviscous effects from the amplitude of the oscillation alone.

We therefore did a few experiments using rather smaller particles in which the magnet was moved suddenly from one angle to another. These proved difficult to interpret mainly because the couple on the particle was dependent on the angle between the magnetic axis of the particle and the field. It thus varied in an uncontrolled manner, and was difficult to measure as it depended on a small difference between two angles. Finally the method of moving the magnet was rather clumsy and difficult to control. We have omitted our results of these experiments, although they did suggest quite strongly that the resistance forces were not characteristic of a simple viscous fluid, and that there might be some elastic effect.

To improve the method it was necessary to use a relatively constant couple, and to apply it suddenly. For this reason we have developed the technique of using single magnetic particles as little permanent magnets, and applying a field, produced electrically, perpendicular to their length.

Our initial experiments, with this method, without photographic recordings, showed that the majority of single particles of the oxidised iron could be moved by the magnetic fields we could apply. They also showed that small fields would often move the particle hardly at all, and that if a large field were applied to a particle for a short time, it appeared to recoil at the moment when the field was switched off. This suggested that elastic effects were probably present. There was also a distinct indication that after the particle had been moved once or twice, it became rather easier to move than before, though it was difficult to demonstrate this unambiguously. Movement of the particles in the absence of an applied field was also noticed. These movements were both rotational and translational; they may have been partly Brownian but they were certainly not entirely so, as they were often not sufficiently random. This feature of the movements of the natural inclusions of the cell, such as small mitochondria, is usually attributed to the streaming of the cytoplasm, though there is some doubt whether this is a sufficient explanation.

2. *Photographic experiments at 37° C*

Early in the course of the work it became clear that to obtain results which we could interpret it was necessary to use only those cells and particles which fulfilled a number of distinct conditions. These have tended to become stricter. They are as follows.

(I) The cell must be a healthy intermitotic fibroblast, from young cultures (24 to 36 hours after sub-cultivation), without large vacuoles and containing few lipoid granules.

(II) The cell must not be far from the edge of the zone of outgrowth so that phase contrast illumination could be used if required.

(III) The cell must not be near other magnetic material, either in neighbouring cells or in the explant.

(IV) The cell must contain only a single particle.

(V) The particle must be of medium size and be fairly short. (The majority of the particles photographed were from 2 to 3 μ in length though one was as small as 1½ μ and one as big as 4 μ. Their shape was usually irregular. Most of them were short, that is, their length/diameter ratio was less than 3.)

(vi) The particle must not be very close to the nucleus or to the side of the cell, nor crowded by cell inclusions.

Preferably it should be possible, with the vertical field, to turn the particle on end, showing that it is not hemmed in by the top and bottom walls of the cell. For this reason very flattened cells were avoided. A spindle-shaped cell was usually chosen with the particle in the middle of the cytoplasm near, but not very near the nucleus.

As might be expected, not many cells fulfil all these conditions, probably on an average about one per culture, so that a fairly extensive search has to be made to find a suitable one. It is difficult, however, to see which condition could be relaxed.

The time-scale of the movements and the magnitude of the angles involved made it difficult to obtain accurate estimates by eye, and it was therefore decided at this stage to use photographic recording.

In our first set of measurements we recorded successfully the movement of particles in 7 cells, though not all of these were entirely satisfactory. The number of records made with each particle varied, but averaged about 10. It is not easy to select with confidence the general features shown by these curves, because there are so many variables, but the following tentative conclusions were reached. The figures (Nos. 4–8) have been selected to illustrate these features.

(1) When the field was switched on, the particles began to move with a fairly high angular velocity. Any initial lag was too small to be detected by our methods. This velocity decreased as the deflection of the particle increased. The extent of the rapid part of the deflection generally increased with the size of the applied field (Text Fig. 5).

(2) After the rapid part of the movement, the angular velocity usually approached a steady value, provided the initial deflection had not been so big that the couple was no longer constant, but on other occasions the particle went more and more slowly, or even stopped (Figs. 4 and 5). In a few cases it appeared to start moving slowly again after having stopped, but the limits of accuracy in measuring the angle made it difficult to be certain of this.

In one case only did the particle start to move very fast after it had been moving steadily, and there were other reasons for regarding this example as exceptional.

(3) When the field was turned off there was, in general, a small fast recoil, followed occasionally by a slow drift in the opposite direction to the original movement (Figs. 4, 5, etc.). This rapid recoil was often of the same size as

Fig. 4

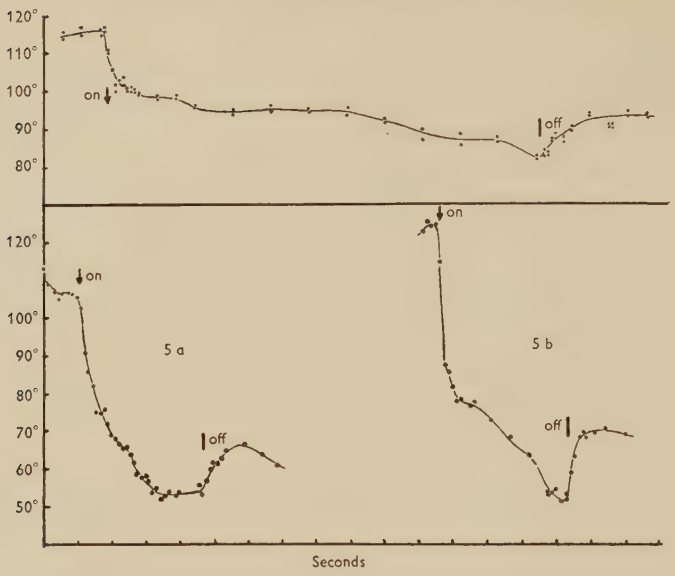


Fig. 4. Response curve of particle in cell. 37° C. 24 oersteds.
Fig. 5. Response curves of particle in cell. 37° C. (a) 8 oersteds; (b) 16 oersteds.

the initial rapid deflection, was never bigger, and was quite frequently smaller. It was sometimes difficult to be certain that it was there at all, especially in cases where it might have been expected to be small, since the marking eyepiece (p. 55) had not yet been fitted, and the natural movements and the experimental error confused the curves.

(4) When a large field was turned on for a short time, a procedure which we call a 'twitch', the subsequent recoil was, in all cases but one, incomplete (Fig. 6). It was usually between $\frac{1}{2}$ and $\frac{1}{3}$ the initial deflection; this could not be accounted for merely by the change of couple with angle. Again there was sometimes a subsequent slow drift which increased the total return.

(5) There was evidence to suggest that if the particles were deflected in one direction, then allowed to recoil, and then, within a few seconds, deflected in the opposite direction to the first, it moved further and possibly more quickly than on the first occasion (see Fig. 7).

(6) In the case of one particle the cell produced considerable natural movements which made it very difficult to obtain consistent readings, and in most of the others there were measurable perturbations (see Fig. 8).

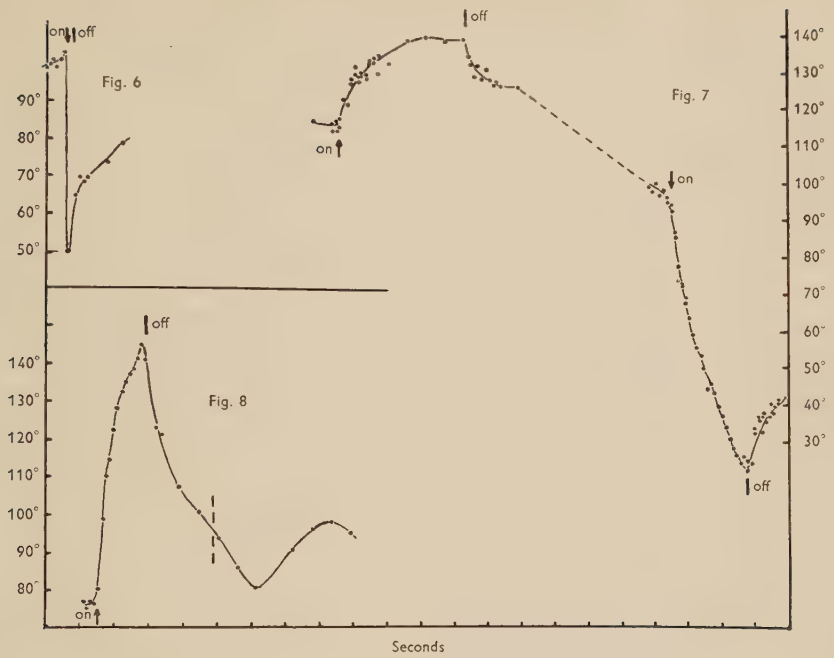


Fig. 6. Response curve of particle in cell. 37° C. 32 oersteds; scale on left.
Fig. 7. Response curve of particle in cell. 37° C. 48 oersteds applied successively in each direction. Scale on right.
Fig. 8. Response curve of particle in cell. 37° C. 32 oersteds. Natural movements follow actuation. Scale on left.

It can be seen from the figures that the results are by no means clear cut. This was partly because the effects of the previous history of the cytoplasm were not clearly realised, and partly due to a general lack of refinement in the technique.

Of these experiments all except one were done without phase contrast, so that it was less easy to be certain of the immediate surroundings of the particle. Moreover the natural movements of the cell were changing the environment of the particle, as well as producing rotational perturbations.

During one of these experiments the temperature had been taken down to room temperature and up again. The results suggested that the physical properties of the cytoplasm at the lower temperature were not greatly different from those at 37° C. The intracellular movements, on the other hand, were very much less active. We decided therefore to undertake a series of experiments at room temperature, and using phase contrast.

3. *Photographic experiments at room temperatures*

These were all done between 15° and 20° C. The cultures were incubated at 37° until required, and were then allowed to cool down, care being taken to avoid the condensation of moisture on the lower coverslip. Measurements were not made until at least half an hour after cooling.

Observation on particles by eye and by time-lapse photography with phase contrast showed that the non-random movements were reduced at room temperatures, but not entirely prevented. Though brownian motion cannot with certainty be detected by eye at room temperatures, the time-lapse films show that there is still some apparently random movement present.

Reliable data was obtained from 4 cells. The average number of recorded actuations for the first 3 was about 12. For the last cell over 50 actuations were filmed. As has been stated the residual field due to the phase contrast apparatus was not negligible in these experiments.

The initial rapid movement, the subsequent rather ill-defined slowing down, the almost universal fast recoil, and the fact that this was usually incomplete were all confirmed. We must make the reservation that when a particle was moving rapidly over large angles, after repeated actuations, it was impossible to estimate the variation of velocity accurately, or to distinguish between genuine slowing down and that due to the couple being less at the extreme angles.

Further evidence was obtained for the effects of repeated actuations. Thus one particle was given a series of twitches, with intervals in between. The results were roughly the same. It was then waggled violently and twitched again. This was done twice. On both occasions it moved faster than in the first series. In addition the recoils were less. Figure 9 shows the effect of twitches before and after waggling.

A further experiment was done with the same particle. After violent waggling with a big field it was allowed some seconds to settle, and was then actuated with a medium-sized field for a few seconds. This was repeated at intervals of the order of minutes. The results showed that over a period of minutes the material appeared to harden somewhat. Figure 10 shows the initial actuation and that 10 minutes later.

Of our other results, for two of the cells we have curves bearing on this point, but although they do not contradict those given in text Figs. 9 and 10, they are open to other interpretations, as the couple was not always constant. Finally we have a series of observations on our fourth cell.

Fig. 9

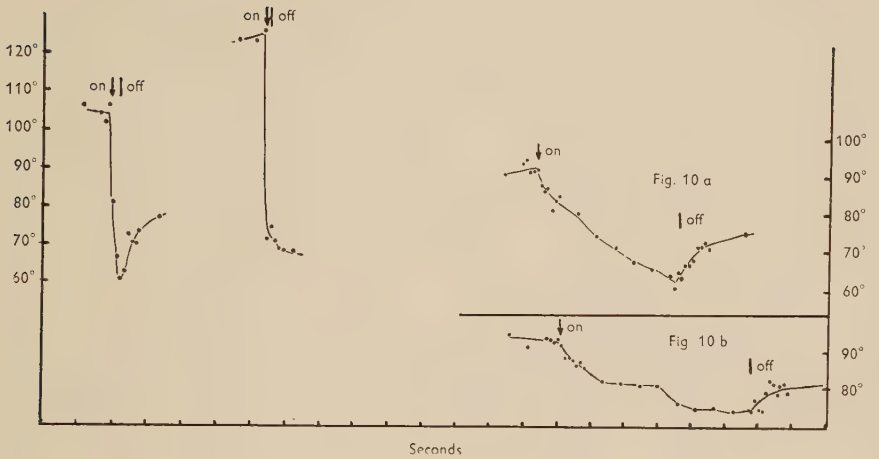


Fig. 9. Response curve of particle in cell. Room temperature. 60 oersteds. (a) before; (b) after waggling. 'Twitch' shorter in (b).
Fig. 10. Response curve of particle in cell. Room temperature. 8 oersteds. (a) immediately after 'waggling'; (b) 10 min. later.

This particle was left undisturbed for 10 minutes before the beginning of the experiment. It was then actuated with a moderate field (about 24 oersteds in this case) for some seconds; after a pause of a second or so, the field was applied in the reverse direction. The cycle was repeated several times until the movements became rapid.

Figure 11 shows the results. It can be seen that repeated actuation undoubtedly allows the particle to move more easily. An attempt was made with this particle to demonstrate the hardening of the material on standing, but the results are too irregular to be worth reproducing, although they suggest that after an 8 minute interval some hardening had taken place.

Our experiments on dragging (see page 70) and our general experience in moving the particles suggested that small stresses often do not produce a yield even if continued for a long time. This is difficult to check at 37° C because of the perturbations, but we have done a few experiments at room temperatures. A typical result showed that for a certain particle $\frac{1}{2}$ oersted for 12 minutes, or 2 oersteds for 3 minutes produced no perceptible yield, whereas 8 oersteds for 45 seconds gave immediate movement followed by a slow yield of about 15°. It was also shown for this particle that even after waggling it gave practically no movement with 2 oersteds, but an immediate movement and a gradual yield with 8 oersteds.

It is not possible to show this behaviour clearly with all particles, especially

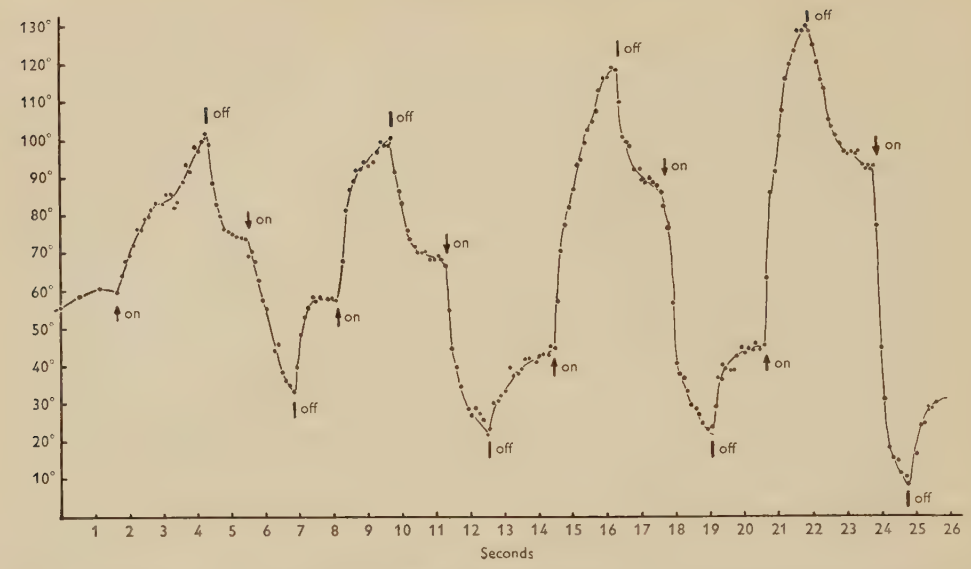


Fig. 11. Response curve of particle in cell. Room temperature. 24 oersteds. Repeated actuation.

if they move under small fields, in which case there are experimental difficulties due to the residual field.

We have made a rough estimate of the order of magnitude of the modulus of rigidity given by our results. Taking as a typical figure a deflection (or recoil) of 12° for a field of 10 oersteds, we obtain

$$n \sim 10^2 \text{ dynes/cm}^2$$

(Recorded values range, all told, from about 20 to 500 dynes/cm^2, but some of this scatter is due to the magnetic material).

The rate of movement after waggling, which can only be obtained very roughly, corresponds to a "viscosity" of the order of 10 poise.

The effect, if any, of boundaries would be to lower both these figures, but almost certainly by less than a factor of 2.

4. *Subsidiary experiments*

Experiments on bias. In interpreting our results we have considered the fast recoil to be a genuine effect, and the slower part to be a possible artefact. To confirm that the recoil is in fact genuine we have devised a special technique which we think rules out all possible magnetic artefacts. This consists

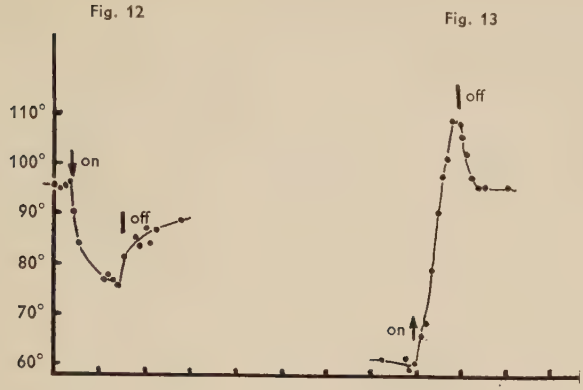


Fig. 12. Response curve of particle in cell. Room temperature. 32 oersteds. Bias: 1 oersted.
Fig. 13. Response curve of particle in cell. Room temperature. 20 oersteds. Bias: 1 oersted.

in applying to the electromagnet a small bias current which remains when the main current is switched off. This bias current can be adjusted to have different values of either sign, and it is thus possible to neutralise or increase any residual field. Examination of numerous cells by eye shows that in general a bias of, say, 1 oersted can prevent or produce an after drift according to its direction, but that there is always, or almost always, a small fast recoil which occurs with either bias. We have made a few recordings of this. Figure 12 +13 show the results for two different cells. The recoil in these cases is certainly genuine, since any magnetic artefact would be less than 1 oersted.

It should be noted, however, that the fact that the bias can prevent a slow drift back does *not* prove that the latter is necessarily an artefact. It may well be that the slow drift is produced by the material near the waist of the particle, which since it has experienced smaller strains will probably be relatively intact. However it can only exert a small couple. If the structure near the ends of the particle had been broken down by the movement, this small couple might be enough to overcome slowly the viscous effect of the broken-down material, and thus produce a slow drift back. This could easily be prevented by a small magnetic field.

Possibilities of this sort greatly complicate the interpretation of experiments with variable stress.

Drifting downwards experiments. At an early stage we had considered the possibility that the particles normally fell under gravity until they rested on the bottom of the cell. We considered this unlikely because the stresses due

to gravity are very small, and the cytoplasm at very low stresses appeared plastic rather than truly fluid. Moreover the movements of the particles due to the cell were often considerable. However to make sure that it was not an important feature we grew some cultures in a vertical position after the first subculture, and after one or two days' growth in the incubator we examined them (at room temperature) in the same orientation. Two cultures were examined, one on the first day and both on the second day. Every intermitotic fibroblast which was growing within 30° of the horizontal and whose outline could be clearly seen was scrutinised. All such cells with particles were roughly sketched. The particles were then grouped into 3 classes, according to whether they were in the top, middle or bottom thirds of the cell (allowance being made for the inversion due to the microscope). The results were:

top third	16
middle third	18
bottom third	15

There is clearly no great tendency for the particles to fall down to the bottom of the cell when the cells are grown sideways.

We have also checked that there is no perceptible drift down under gravity after the particles have been waggled violently in all directions. This was also done by fitting up a microscope horizontally, so that the cultures could be mounted vertically, and single particles in spindle-shaped cells were observed. The experiments were done at room temperatures.

Five cells were observed. One was rejected because the particle showed large angular perturbations due to the cell. Two showed no detectable movement during the time observed, which was 20 minutes from the cessation of waggling. One showed no movement for 10 minutes, and then moved *upwards* about 1 μ in a few seconds. The last one showed a slight brief upward movement after about 5 minutes, but no further movement in the remaining 15 minutes. Thus, as expected, there is no steady drift downwards, but at these temperature (20° C) the natural movements of the cell are not entirely suppressed.

BIREFRINGENCE

We have made a brief attempt to detect birefringence in the cytoplasm in the neighbourhood of a twisted particle. This was done with Mr. J. M. Mitchison of the Zoological Laboratory, Cambridge, on his polarising microscope, for which assistance we are very grateful. The phenomenon was

complicated by polarising effects due to the particles themselves. This was confirmed by observing the particles in a mixture of equal parts of glycerine and water. It was therefore difficult to observe small amounts of birefringence near the particles, which is the only place in which we should expect it.

The experiments were done at room temperatures. Although several cultures and a number of cells were tried, some with fairly large particles, no effect could be detected.

A negative result is not really surprising because if there were an effect it might be expected to be small.

5. *Sodium oleate and bentonite*

We have made some observations at room temperatures on magnetic particles in two materials of known composition. The same technique were used as for the previous experiments, except that rather larger particles were chosen in order to increase the accuracy.

The first material was an elastic soap solution, very kindly supplied to us by M. Dervichian of the Institute Pasteur, Paris, and prepared by him as follows.

Concentrated soap solution:

36.0 gr Na-oleate
180 cc KOH 2 N.
820 cc H_2O

KCl solution:

3.8 N (that is, 283.3 gr/liter)

The elastic soap solution itself was prepared as follows.

20 cc. Concentrated soap solution
22.5 KCl solution
17.5 cc H_2O

Thus the final concentration of sodium oleate was about 1.2 per cent by weight.

The results showed that when the magnetic couple was applied, there was an initial rapid deflection, followed by a constant rate of yield. When the field was removed, there was a swift recoil equal in size to the initial fast deflection. This is illustrated in Text Fig. 14 a. Moreover both the recoverable deflection and the rate of yield were approximately proportional to the applied couple. The "relaxation time" was about 0.9 seconds.

Fig. 14

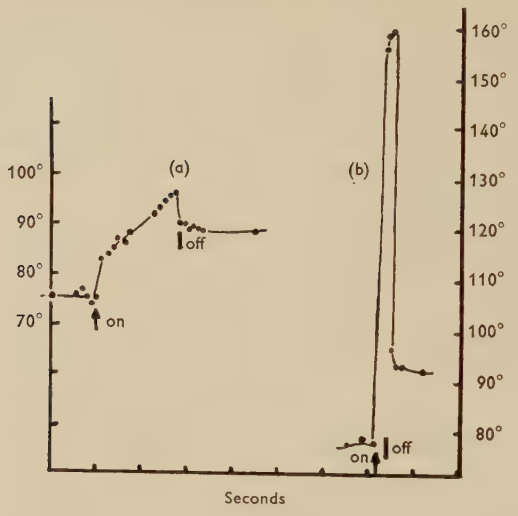


Fig. 14. Response curve of particle in sodium oleate. (a) 3 oersteds; (b) 24 oersteds.

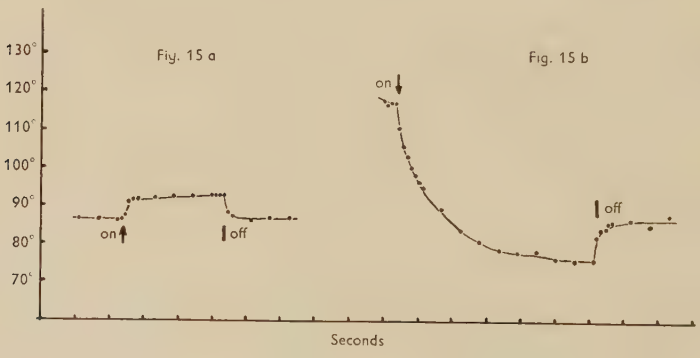


Fig. 15. Response curve of particle in Bentonite. 8 oersteds. (a) before; (b) after 'waggling.'

The effect of a large field for a short time (a twitch) gave a high degree of recovery even for large angles, as illustrated in Fig. 14 b.

The second material was a dilute gel of the clay, Wyoming Bentonite, given to us by the kindness of Dr. M'Ewen and Mr. Mould of King's College, London. It had been roughly fractionated by centrifuging. The particles are believed to be thin plates, which could not be seen by the phase microscope. The concentration was about 1.8 per cent by weight, and no extra

salts or NaOH had been added, so that the material was a feeble thixotropic gel.

After having been allowed to stand, this material also gave a swift initial response, which was approximately proportional to the applied couple. The modulus of rigidity was $1\frac{1}{2} \times 10^2$ dynes/cm^2 approximately. If the couple were small, there was little or no yield, but if the couple were increased, the yield went up rapidly. Thus, in one case, $\frac{1}{2}$ oersted for 16 minutes, or 4 oersteds for 2 minutes, gave no significant permanent deflection ($< 2°$), whereas 8 oersteds for 1 minute gave a yield of 6°. Repeated actuations by large couples appeared to destroy the gel structure, so that small fields, which initially gave small recoverable deflections, would give large rambling permanent ones. (See Fig. 15.) On standing, the gel would harden again and a series of small actuations taken 5, 10, 20 and 40 minutes after vigorous stirring show increased hardening at each stage.

We have not succeeded in twitching a particle through a large angle without producing a permanent deflection. When the particle was deflected through a large angle in one direction, so that a big yield was produced, it moved even more when the couple was reversed, as might be expected on theoretical grounds, since in effect it had made the beginnings of a small hole for itself in the gel, through which it could move back easily on reversal until it came near fresh unsheared material.

A few measurements on a gel where concentration was threequarters that of the first gave substantially the same type of results, though the coefficient of elasticity was rather less than before, being roughly 0.3×10^2 dynes/cm^2. Material with only half the original concentration did not gel when put on the slide, and showed Brownian motion.

Thus under the conditions we have used these two materials show behaviour of a very different character, although their elastic moduli are of the same order of magnitude. These differences are, in fact, quite obvious in the test tube. The soap solution, when shaken, shows lightly damped elastic oscillations. When poured it pulls out into long semi-liquid filaments. On the other hand, the bentonite hardens into a rigid gel, which is easily destroyed by tilting the test-tube. Probably the best way to describe the difference between them is to say that the "relaxation time" of the bentonite is a function of stress, while that of the elastic soap solution is roughly independent of it.

Though these experiments are of little value for finding the structures of these substances, they do establish experimentally several points about the behaviour of twisted magnetic particles in different types of material.

F. DRAGGING

1. *Biological technique*

As stated above, for our dragging experiments specially small culture vessels were devised. The cultures were made in the usual way, with magnetic material in the medium, but they were subcultivated twice. On the second day of the last passage they were transferred to specially prepared chambers.

Each chamber was made from a strip of platinum 0.25 mm thick, about 2 cm long, and 1 or 2 mm wide. This strip was bent round, a glass rod being used as a jig, into the shape shown in Fig. 16, and then fixed together at

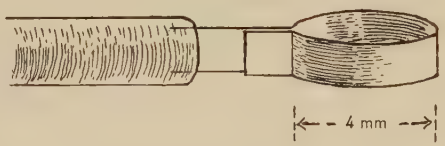


Fig. 16. Diagram of mount for small cultures.

the join with a small piece of glass, by heating in a bunsen flame. A glass rod was then fused to the free end of the platinum strip to act as a handle. The top and bottom surfaces of the platinum chamber were made approximately flat by rubbing them on fine emery paper on a flat surface.

The small coverslips, about 4 mm diameter, were cut from ordinary No. 1 coverslips with a diamond. A hole in a piece of perspex was used as a jig. The top coverslip, on which the culture was to be mounted, was fixed to the ring with paraffin wax. This was done by dipping the platinum into hot wax, after which it was withdrawn shakily to remove surplus wax, and allowed to cool. The coverslip was placed lightly in position, and the whole warmed over a hotplate till the wax melted and ran into the corner round the edge of the coverslip. The hot plate was then turned off, and the wax allowed to cool very slowly, so that large crystals were formed. It was found that the seal produced by quick cooling was unsatisfactory, and allowed water vapour to leak out, but the slowly-cooled wax was water-tight. A plastic material was also tried. This was satisfactory, but it was more trouble to prepare than the wax, as it had to be baked.

For the lower coverslip which was not in contact with the plasma clot, vaseline was used as a seal, as this joint had to be made with the living culture in position. The platinum chamber and the coverslips were sterilised *before* waxing, and thereafter kept under sterile conditions.

The technique for the final subculture, which was done for us by Dr. Honor B. Fell, was as follows.

The chamber was mounted upside down, so that its single coverslip formed a floor. The explant, instead of being divided into two, was cut into five or six pieces, each about $\frac{1}{2}$ mm square. Originally each tiny fragment was added to the usual mixture of embryo extract and plasma, and then pipetted into the small chamber. This was rather a race against clotting, and it was found more satisfactory to pipette the explant in with the embryo extract and add the plasma afterwards. Any excess was then removed with another pipette. The technique was tricky, but there were only occasional failures.

When the medium had clotted, vaseline was smeared round the lip of the chamber and the other coverslip added. The chamber was then turned the right way up and put in the incubator.

2. *Apparatus and calibration*

Our experiments were made with very simple apparatus. The U-shaped Alnico magnet described previously (page 47) was used with a single soft-iron pole-piece of tapering shape. Several types were tried, and using a simple test we selected the one which gave the greatest force. The test consisted of finding the force on a small speck of the magnetic material embedded in a blob of glue and fixed to the end of a glass filament. The deflection of the filament and the distance of the magnetic material from the pole face were observed under a low power (2/3″) microscope, and measured with a micrometer eye-piece. This method enabled rough relative measurements to be made, giving the curve of force v. distance from polepiece, and enabling difference pole-pieces to be compared. A crude estimate of the absolute value of the force per unit volume of the magnetic material was made by calibrating the filament with a 10 mg weight, and estimating the volume of the material. This gave the force per unit volume as about 2×10^6 dynes/cc at about $2\frac{1}{2}$ mm distance, but this figure should probably be increased by a factor of 2 to allow for the real volume being less than the apparent volume. For a more accurate calibration it would be necessary to record the velocity of the particles in castor oil.

3. *Experiments*

Once the initial difficulties with sealing had been overcome the cultures grew quite well, although the tissue deteriorated rather more quickly than in the usual preparations. The material was, as usual, frontal bone of 11 or 12 day chick embryos, incubated at 37½ C. The culture was examined by means of a ×20 objective and a ×15 eyepiece. The magnetic particles could be seen very clearly, and also the general shape of cell, though naturally not all the finer details.

The magnetic field was applied by simply sliding the magnet into position along the stage. This had the disadvantage that it could not be done suddenly. The pull was horizontal; that is, parallel to the cover-slip. No particles were observed to move quickly. Some particles never moved at all during about half an hour's observation. A few particles could be seen to be moving if watched carefully. A typical rate of movement was the particle's own length in half a minute. The movement was not always regular. We recorded two sequences photographically.

These results were much as expected, as the stresses applied were low. To obtain better results the pole-pieces should be redesigned and made of more suitable material to increase the gradient, and that the field should be produced by an electromagnet, so that it could be switched on and off suddenly. This we have not yet done.

G. PRODDING

We record these observations for the sake of completeness but we have not developed the technique very far.

As has been stated, we apply in this case a fairly large field of about 600 oersteds. The cultures are made with a relatively large quantity of magnetic material in the medium cells containing many particles are chosen. Under favourable circumstances the particles can be lined up by the field and if this is done with the field parallel to the length of the cell, a composite rod of length somewhat greater than the width of the cell can sometimes be formed. If this rod is rotated by rotating the magnetic field it will eventually come up against the walls of the cell. Further rotation frequently causes the rod to break up into its component parts, but occasionally we have succeeded in making it push out the cell wall. It can be made to protrude some distance without apparently causing any break in the wall. It was not pos-

sible to see any displaced membrane, even under phase-contrast. If the magnetic field were then reduced in size without its direction being altered the particle appeared to be pushed back by the cell wall.

We have also been able by a similar technique to prod the nucleus inwards. This is not easy to do, and still more difficult to establish that one has done it. The only conclusive test seems to be the movement of adjacent nucleoli. This we have observed on several occasions.

It is obvious that the forces that can be applied by this method are considerably bigger than in our usual twisting technique, but we have not as yet evaluated them.

H. DISCUSSION

1. *Previous work*

We do not propose to review in detail all the literature on protoplasmic viscosity. A fairly recent account has been given by Heilbrunn (An outline of general physiology, 2nd Edition, 1943). His monograph (The colloidal chemistry of protoplasm, 1928) covers the earlier work in more detail. It will suffice to say that in certain materials, such as the protoplasm of the sea-urchin egg (*Arbacia*), and the interior of *Amoeba dubia*, the viscosity is not many times that of water, while in other materials, such as the ectoplasm of *Amoeba proteus*, the cytoplasm appears to be more in the nature of a gel.

We will consider, mainly, previous work in which the magnetic particle method is applied. Its first recorded use is in the classical experiments of the botanist Heilbronn, who inserted small pieces of iron into slime moulds. These are discussed by Heilbrunn (1928, p. 62). It appears to us, if we have understood the description of the experimental technique correctly, that whatever else was being measured, it was certainly not the viscosity. To measure viscosity it is necessary to measure a *rate* of movement, whereas Heilbronn found the field that would just move the particle. Moreover, although he gives no details of the magnetic properties of his iron rods, it is very probable that the forces he applied would have moved freely suspended particles very rapidly, especially in the calibration experiments in water and ether. These experiments did, in fact, show unexplained deviations. There seems little doubt that the particles were resting on the bottom, and that Heilbronn was measuring something which might be called adhesion.

Both Heilbronn and Heilbrunn discuss this possibility of adhesion, but they do not seem to have realised that in all probability such forces were

entirely swamping any effect due to viscosity. The difficulty would have been avoided if an estimate had been made of the absolute value of the couples applied, instead of relying entirely on a "calibration" in known fluids.

It is all the more remarkable that such consistent results should have been obtained, and a repetition of their experiments might be well worth while.

Freundlich and Seifriz (1922) inserted nickel spheres into gelatin and other gels, and attracted them with an electromagnet. These experiments are unobjectionable, except for the fact that these workers also made no estimate of the absolute values of the properties measured. The gels used were probably rather stiffer than those on which we have experimented. Seifriz's attempts to apply the technique to the egg of the Sand Dollar *Echinarachinus* (Seifriz, 1924) — were less fortunate and Heilbrunn's criticisms here are more to the point. Although Seifriz claimed to have demonstrated an elastic cortex, it is not certain that the observed recoil was not due to the cell membrane. Moreover, he probably damaged the egg while inserting the nickel ball. His estimate of the interior viscosity lacks precision.

Pfeiffer (1936) has made experiments in which protoplasm has been forced through a capillary tube, and has shown that the "viscosity" varies with the rate of shear, but here again it is not clear how much of the effect may not be due to the pellicle or to the damage produced by rough handling.

Many workers have shown that different structures such as the erthyrocyte membrane, or giant chromosomes of the salivary glands of Dipterous larvae, are elastic, probably highly so, but their properties are not directly relevant to the question of the elasticity of the cytoplasm itself. We include in this category the spindles and asters of dividing cells. To use the term protoplasm in a loose way to cover all these materials as well as the hyaline cytoplasm, as Seifriz does (1936, page 233) may occasionally be convenient, but it makes nonsense of any discussion of the structure of the cytoplasm.

No quantitative work appears to have been done on vertebrate tissue cells, although some have been centrifuged (Beams, 1943, p. 71) and some have been microdissected (Chambers 1924). Chambers and Renyi (1925) in a study of vertebrate epithelial cells by microdissection state "In consistency the protoplasm of the different cells studied varies from the very tough, jelly-like solid of the squamous epithelial cell to the very fluid cytoplasm of the gland cells." It is probable that the differences observed in microdissection do correspond to differences in the physical properties of the cytoplasm, but it is not possible to make the results as they are stated, even approximately quantitative.

2. *The present experiments*

We are now in a position to survey the results of our experimental work. We have established that when a magnetic particle is deflected in the cytoplasm of a chick fibroblast

(1) there is usually a small fast recoil;
(2) this recoil is usually incomplete, and always so for very large angles of twist;
(3) the order of magnitude of the modulus of rigidity is 10^2 dynes/cm^2;
(4) particles can frequently be made to move more easily after repeated movement;
(5) some particles cannot be moved appreciably by very small stresses, even if applied for a long time.

We have only attempted to demonstrate the last point at room temperatures, and not at 37° C.

The question now arises: are these effects due to the cytoplasm itself, or to other factors, such as the cell inclusions or boundaries?

We have argued (page 42) that the results are not due to the particle being in a vacuole, nor do we think that they can be produced by the proximity of the natural inclusions of the cell. It is possible that the elastic recoil is due to the cell membranes, but we think it improbable. Our experiments on bentonite encourage us to attribute the phenomena to the cytoplasm itself, which we believe to be a thixotropic gel, showing feeble elastic properties.

There is little new in these conclusions. It has been advanced for many years, though often on rather inadequate evidence. However we believe that this is the first time that the order of magnitude of the elastic modulus has been established. The value shows that the gel is very feeble, so feeble in fact that in bulk in a test-tube it would probably be classed as a sol or an elastic liquid rather than a gel.

It is difficult to interpret point No. 2 above because of the complications involved when measurements are not made at constant shear. The results certainly suggest that chick cytoplasm may not be "highly elastic", in contrast to the sodium oleate solution, and that the relaxation time is a function of the shear. In this respect it appears to resemble the very feeble gel of bentonite, though the analogy should not be pressed too far, as the similarity may not extend to all features of the behaviour.

It is often assumed that normal cytoplasm is "highly elastic" but it is doubtful if there is any really convincing evidence for this. What has some-

times been observed is the elasticity of surface membranes. Our observations on prodding, and all other experimental evidence, suggest that in these chick cells the physical properties of the surface membrane are quite different from those of the interior.

Attempts to demonstrate highly elastic behaviour by drawing out strands of cytoplasm are open to the criticism that in doing so a new surface layer is formed, and that this, rather than the cytoplasm itself, may be giving the effect.

It is probable that a quantitative investigation of certain other biological gels, such as the ectoplasm of *Amoeba proteus*, would show elastic properties of a similar type. Indeed the centrifuge data suggest that the elasticity of this material is rather weak (Heilbrunn, 1943, p. 67).

Incidentally it should be noted that our technique is not sufficiently precise to enable us to say whether the inside of the chick cell is uniform in its physical properties. For example, we cannot rule out the possibility that the middle of the cell may be rather more fluid than the regions nearer the cell membrane.

Can we, from our results, deduce anything about the underlying structure of the cytoplasm? The analogy with bentonite is in some ways fortunate, because nobody will be tempted to think that the cytoplasm *is* bentonite; but we must consider whether there is a real content in the analogy.

At present the only conclusion is that dilute gels cannot show elastic properties unless they contain highly asymetrical particles or aggregates. This is probably true. For more concentrated solutions, such as cytoplasm, it is not clear how far this idea can be applied. Presumably the more "highly elastic" the material is, the higher is the assymetry required.

At small stresses, the approximate constancy of relaxation time with stress probably indicates a purely thermal relaxation, while for materials which are relatively plastic the relaxation is probably due to the shear itself. This suggests that in the latter case the shear is carried by fewer links than in the former instance, assuming that in both there is roughly the same temperature dependence. However our results are for comparatively large strains, and perhaps reflect more the configuration of the materials.

The difficulty here is that there is as yet no general theory of the structure of substances of this sort, so that it is not yet possible to relate features of their behaviour with features of their structure. Much work has been done on rubber-like substances and gelatin gels on the one hand, and dilute solutions of very large molecules on the other, but no studies on elastic liquids, other than of an exploratory nature, have yet been published.

One approach to this problem might be to take certain biological materials,

such as crude protein solutions, or possibly a suspension of Claude's microsomes (Claude, 1943) etc., and see how magnetic particles behave in them. We believe this method might lead to misleading interpretations due to superficial similarities in behaviour. It is important to discover which features of a model are essential to give the properties measured, and which are accidental. To do this it is necessary to have a theory, however crude, to account for at least some of the observable features.

This can best be constructed by using, in the first instance, chemically defined materials in test-tube quantities, so that a large variety of methods (birefringence, light scattering, X-rays, etc.) can be brought to bear on each particular substance. There is then some chance that the structure can be determined unambiguously, so that there will be a firm foundation for any theory.

When this has been done, it might be profitable to reinvestigate the properties of the cytoplasm. It is unlikely that all the methods available for test-tube amounts could be used on the cytoplasm, both because of the size of the cell and also owing to the biological limitations. However, from the limited data which could be obtained it might be possible to establish some features of the structure. The magnetic particle method might prove very valuable at this stage.

We must consider the theories of cytoplasmic structure which have been put forward in the absence of such an approach. Of course it may well be that no general theory is possible; different biological materials may differ so radically that their cytoplasm has little in common.

If we neglect hypothesis of merely historical interest, there are at present two main theories of cytoplasmic structure: the "brushheap" theory of Seifriz (1936, p. 247) and the "frame work" theory of Frey-Wyssling (1948). Both are necessarily vague because of the absence of precise data and where they attempt to be more definite they are open to criticism. For example Frey-Wyssling states "The members of the fibrous framework are extraordinarily thin: the order of magnitude of their thickness is that of the molecular cross-section of a single polypeptide chain". This may be true, but there does not appear to be any evidence for it. The argument really amounts to saying that they cannot be so thick that they can at present be detected. Does he regard 50 Å, for example, as being of the order of magnitude of a *single* polypeptide chain?

Nor is it obvious that the elements of the framework must necessarily join to each other as postulated by Frey-Wyssling. In a fairly concentrated solution, they may be linked more often by the other molecules present. Finally although presumably there must be some asymetrical units present, it

is not obvious that they are necessarily flexible. Are bentonite plates flexible, for example? This brings us to a picture rather like Seifriz's brush-heap, which, if we understand correctly the implication of his photograph of a pile of matches, contains rigid rodlets.

It is clear that if these two models are compared, there is no real evidence on which to decide the points on which they differ. All that can reasonably be said is that in the cytoplasm of some materials there are probably asymetrical units or aggregates present, which are not large enough to be easily detected.

If we were compelled to suggest a model we would propose Mother's Work Basket — a jumble of beads and buttons of all shapes and sizes, with pins and threads for good measure, all jostling about and held together by "colloidal forces".

Finally we would plead that the evidence we have presented for elasticity, and therefore a "structure" in the cytoplasm will not be regarded as evidence for some "cytoskeleton" in the chick cell. This implies, as we understand it, a fixed and characteristic spacial relationship between the highly specific components of the cytoplasm, extending throughout the cell. Such evidence as we have is against such a conception as applied to the cytoplasm as a whole (in contrast to individual parts of it, such as the mitochondria) since the cell produces considerable relative movement of many of its natural inclusions, especially at 37° C.

We particularly deprecate statements such as that of Frey-Wyssling (loc. cit. page 115) that "the structure of plasm must be of a wonderful coordination. The framework cannot represent an unordered pile, but must possess an organised and well-defined structure". We do not see why the presumably coordinated chemical actions of the cytoplasm *must* imply an *organised* frame-work in the cytoplasm, except possibly in special cases. The "structure" we have established may well be a rather non-specific, transient affair for a normal cell.

3. *The magnetic particle method*

We summarise here briefly the advantages and disadvantages of the magnetic particle method as they appear to us.

The paramount advantage is that it is possible to apply a stress which varies swiftly with time. This has enabled us to distinguish small elastic effects which would be difficult to demonstrate by any other method.

Secondly, the stresses can be applied locally; that is, it is not necessary to apply them intensely to the cell as a whole, as in centrifuging.

Thirdly, the stresses in the case of twisting, and also for the dragging of large particles, can conveniently be made high, and for twisting, though less conveniently, very high.

Among the disadvantages are the difficulty of getting the particles into the cell, since only certain types of cells will phagocytose them; the possibility that they may modify the neighbouring cytoplasm; and the care needed to perform some of the magnetic measurements without artefacts. For small particles the accuracy is low, though this could probably be improved for larger particles. The method of recording and analysing the results is rather laborious.

The fundamental disadvantage that the properties cannot be measured at constant stress applies to all methods for living material with which we are familiar.

ACKNOWLEDGEMENTS

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SUMMARY

1. A magnetic method is described for the study of the consistency of the protoplasm of living cells in tissue cultures.
2. Such protoplasm is not purely viscous, but has elastic properties.
3. The order of magnitude of the modulus of rigidity is 10^2 dynes/cm^2.
4. There is evidence of thixotropic behaviour.
5. The bearing of these results on current theories of protoplasmic structure is discussed.

APPENDIX

In order to understand the meaning of the terms used, consider text Fig. 17. This represents the deflection of a body in a viscoelastic medium, plotted against time, when a constant stress is suddenly applied and later suddenly removed. If the viscosity were very small, the deflection would go from A to B when the stress was first applied, and AB would represent the recoverable, elastic, deflection. The actual viscosity, however, delays this, and so the deflection follows AC. When the stress is removed, at D, then deflection DE (equal to AB) is recovered. Viscosity again delays the response. There has been, during the time the stress was applied, a steady, irrecoverable

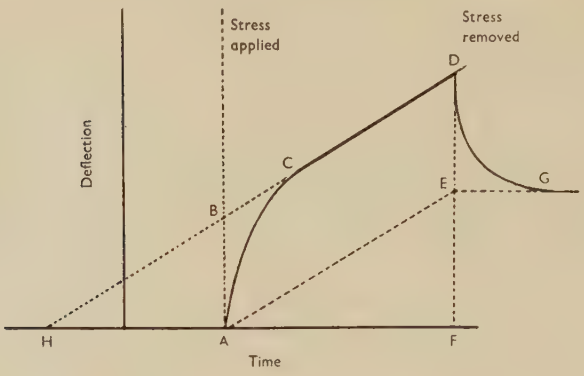

Fig. 17. Explanation of visco-elastic yield (see Appendix).

yield, and this has amounted in all to the deflection FE. This yield has been called the pseudo-viscous yield.

The relaxation time is the distance HA. A simple way of thinking of it is as follows. Suppose that a stress is applied to give a recoverable, elastic, deflection (AB) of 5°. Then suppose that if this same stress is maintained for t seconds the pseudo-viscous yield (EF) also just amounts to 5°. Then the relaxation time is t seconds. The faster the yield rate, the smaller the relaxation time.

It can be seen that if we apply, say, double the previous stress, and if both AB and EF are just doubled (i.e. the initial recoverable deflection is twice as big, and the pseudo-viscous yield twice as fast), then the relaxation time, HA, is the same as before. In some materials, however, although AB us doubled, EF is much more than doubled, so that the relaxation time is then not constant with stress.

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THE DISTRIBUTION OF MASS AND LIPIDS IN THE SINGLE NERVE FIBER

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I. INTRODUCTION

The present investigation is a part of a study in which quantitative cytochemical methods are applied to the analysis of the chemical composition of the single nerve fiber in the resting stage and after stimulation. This paper deals mainly with the distribution of mass and lipids in the non myelinated nerve fiber. The ultrastructure of the axon will also be discussed briefly. A preliminary note on the findings has already been published by Engström and Lüthy (1949).

II. MATERIAL

Single myelinated nerve fibers were obtained by microdissection of the sciatic nerve of the frog (cf. Stämpfli 1946, Lüthy 1948). Non myelinated fibers were obtained from the olfactory nerve in the pike. About 50 preparations of myelinated fibers were made but in the case of non myelinated nerve fibers only a few preparations were performed.

The fibers were dissected in Ringer solutions under the dissecting microscope using dark field illumination. After the single fibers were obtained they were rapidly washed in distilled water. The muscle fibrils and the undissected part of the nerve were cut off after the single fibers had been placed on the metal holders used for the X-ray examination (see below). During the dissection checks were made to establish that the fibers were conducting. The preparations were allowed to dry at low temperature in a refrigerator and controls revealed that the fibers did not change form very much or collaps during the drying procedure.

III. METHOD

The method used for the determination of the mass of different parts of the nerve fibers was the one described by Engström and Lindström (1949). In that method a microradiogram is taken of the biological specimen and a reference system built up of strips of nitrocellulose. The X-ray absorption of the specimen is compared with that of the reference system. Soft X-rays, 3,000 to 4,000 volts, filtered in 9 μ Al, giving a wavelength band of about 8–12 Å, were used. The Al-foil is necessary for filtering out shorter wavelengths, below 8 Å, which give an error in the mass determination.

A consideration of the theory of the X-ray absorption revealed that the mass of a structure in biological material might be determined with an error of less than 5 per cent even if there are relatively large variations in the proportion of the constituent elements. The basis of the calculation is the assumption that the tissue consists only of carbon, oxygen and nitrogen in the same proportions as in protein. The mass absorption coefficient for this mixture is calculated. The biological tissue, however, consists of a number of elements in addition to those mentioned. To the basic protein mixture of C, N and O other elements including C, N and O independent of protein, are added and their influence on the mass absorption coefficient calculated. An element with a high atomic number has a greater absorption than corresponds to its contribution to mass. The influence of the different elements is calculated for different wavelength regions.

Fig. 1 shows the allowable percentage of the elements which gives a total systematic error of 5 per cent in a 3,000 volt X-ray spectrum filtered in 9 μ Al. If, for example, the concentration of Cl is 14 per cent, that of Na 2 per cent and that of K 2 per cent the error produced is only 5 per cent. The only element in ordinary tissues that must be corrected for as seen in fig. 1 is H. Using the final formula 10 a and the precautions given in the paper by Engström and Lindström (1949) the mass of a cytological structure can be determined by the X-ray absorption technique with an over-all error less than 10 per cent.

The diameters of the fibers were measured on the enlarged microradiograms. The microradiograms of the fibers and reference system were enlarged 200 to 500 times by photomicrography. The photometric comparison between the X-ray absorption in the fibers and the reference system was performed on these enlarged microradiograms, following the procedure described in the quoted paper. The absorption of a certain part of the

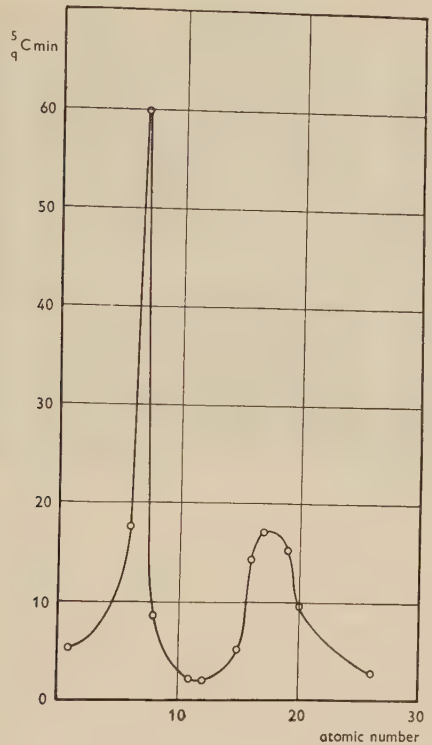
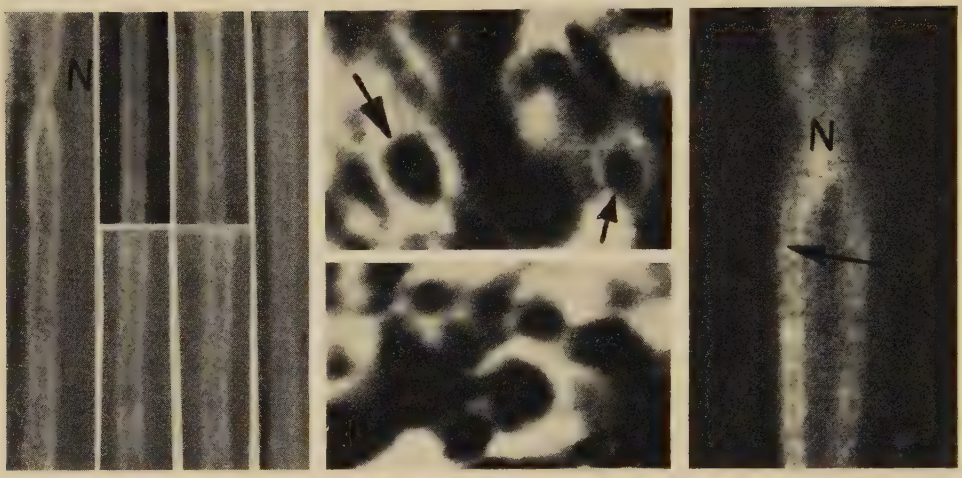


Fig. 1. The allowable percentage of the elements which give a systematic error of 5 per cent in an X-ray spectrum with the wavelength region 8–12 Å. (From Engström and Lindström 1949.)

specimen was obtained in equivalents of the reference system. Knowing the mass per unit area and the chemical composition of the unit in the reference system the absorptions measured in the fibers could be corrected to mass per unit area.

IV. RESULTS

In Fig. 2 are shown enlarged microradiograms of single nerve fibers. To the left (2 a) myelinated fibers are shown and the X-ray absorption of the outer part of the fibers is stronger than that of the central parts. The outer, strongly absorbing portion, corresponds histologically to the myelin sheath, and the central portion to the axon. The absorption of the myelin increases in the neighbourhood of the nodes of Ranvier (N), which also can be seen



a. b. c.

Fig. 2. a, enlarged microradiograms of single myelinated nerve fibers. The white areas are those absorbing X-rays. N indicates a constriction of Ranvier. Magnification c. 300 x. b, cross section of nerve fibers. The arrows indicate the myelin sheath of some cross cut fibers. Magnification c. 800 x. c, part of a single nerve fiber after extraction with lipid solvents. The myelin has been extracted in the center, indicated by the arrow. N is a constriction of Ranvier. All pictures are taken with 4,000 volt X-rays filtered through 9 μ Al. The main intensity in the X-ray spectrum used lies between 8 and 12 Å.

in the picture. In the nodes no cross membranes or similar structures could be observed. In Fig. 2 b is shown a cross section of a bundle of fibers indicating the low X-ray absorption in the axon when compared with the myelin. Fig. 2 c shows a myelinated fiber after lipid extraction and the central part of the myelin sheath has lost much material.

Fig. 3 shows a schematic cross section of a myelinated fiber. The probable cross section of the dried fiber is discussed later in this paper. The X-ray absorption was measured at A and B. The outer diameter is 2R and the inner 2r. 2r thus is the diameter of the axon. B : 2X is the X-ray absorption equivalent per μ myelin and $\frac{A - \frac{B}{x}(R - r)}{2r}$ is the X-ray absorption equivalent per μ axon. Measurements from ten fibers are shown in Table I. The absorption per μ myelin is 0.11 foil equivalents and that of axoplasm 0.028 foil equivalents. The unit in the reference system had the weight 234 μg/cm² = 2.34×10^{-12} g/μ². One μ³ myelin thus corresponds to 0.268×10^{-12} g of foil. The correction for H in the sample and reference system, together with all correction factors (see Engström and Lindström 1949) is 1.1–1.2.

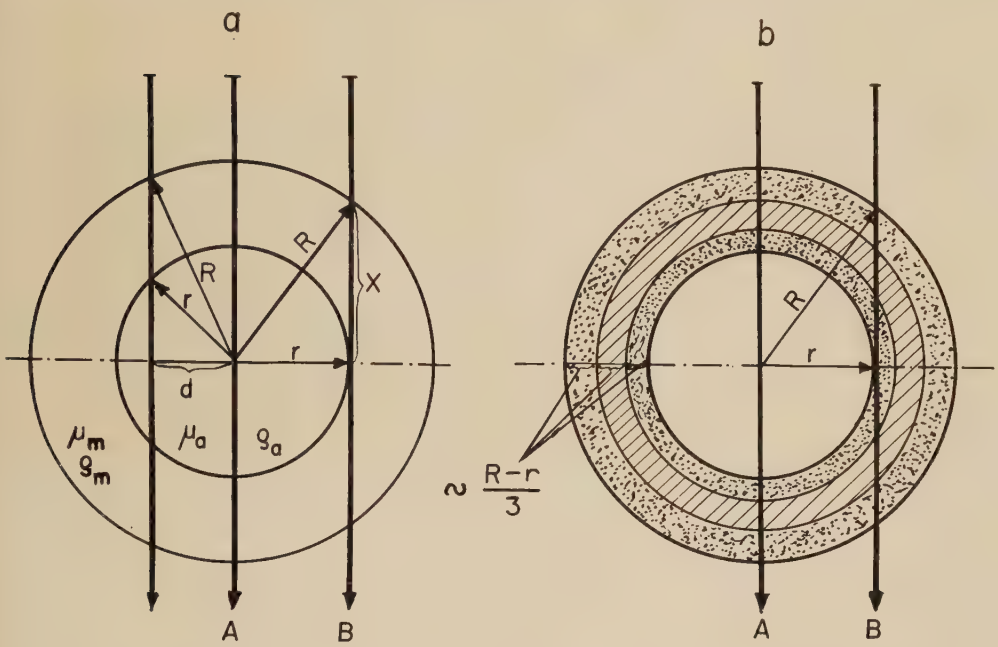


Fig. 3. a, schematic cross section through a myelinated nerve fiber. The X-ray absorption is measured at A and B. (R-r) is the thickness of the myelin sheath. b, schematic cross of a nerve fiber after lipid extraction. The hatched area in the center of the myelin sheath is the one which loses most material when extracted.

The weight of the myelin thus is 0.3–0.4 $\times 10^{-12}$ *g/*μ^3. If the specific density in the living state of the fiber is assumed to be about 1 the myelin sheath contains 30 to 40 per cent dry substance.

The axon has the uncorrected weight 0.0655×10^{-12} g/μ^3. The correction factor is 1.1–1.2 which gives the weight $0.076–0.089 \times 10^{-12}$ g/μ^3. *The axon thus has about 8–9 per cent dry substance.*

The nerve preparations were extracted on the sample holders with alcohol ether and petroleum ether for 72 hours. Measurements of mass were performed and the results are shown in Table II. The myelin now has the absorption 0.091 foil equivalents per μ. A loss in mass in the ratio 9 : 11 thus was observed. The axon, however, showed a mass of only 0.013 foil equivalents if the mean is calculated on all four measurements. If the three positive measurements are taken into account the mean is 0.017 ± 0.0002. At first sight there appears to be a considerable loss of mass (high lipid content) in the axoplasm. However, on the pictures it could be observed that only the central part of the myelin sheath is extracted. This is schematically

Table I

2 R μ	2 r μ	2 (R − r) μ	2 X μ	A	B		B: 2 X		$A-\frac{B}{X}(R-r)$ / 2 r
12.5	6.4	6.1	10.8	0.80	1.30	1.50	0.12	0.14	0.002
16.0	7.2	8.8	14.3	1.28	1.38	1.70	0.10	0.12	0.043
14.0	5.3	8.7	13.0	1.26	1.42	1.42	0.11	0.11	0.057
10.0	5.0	5.0	8.6	0.70	1.00	1.04	0.12	0.12	0.020
12.5	6.5	6.0	10.7	0.75	0.98	0.98	0.09	0.09	0.032
12.5	5.7	6.8	11.1	1.00	1.24	1.40	0.11	0.13	0.035
13.5	7.2	6.3	11.4	0.60	0.85	0.70	0.07	0.06	0.026
11.5	4.5	7.0	10.6	0.80	1.36	1.28	0.13	0.12	—
15.0	6.8	8.2	13.4	1.23	1.70	1.40	0.13	0.10	0.043
14.0	8.2	5.8	11.4	1.00	2.04	1.24	0.18	0.11	0.020
							0.11 ± 0.006		0.028 ± 0.006
							$\sigma = 0.025$		$\sigma = 0.019$

Table II

2 R μ	2 r μ	2 (R − r) μ	2 X μ	A	B		B: 2 X		$A-\frac{B}{X}(R-r)$ / 2 r
15.0	9.0	6.0	12.0	0.75	1.00	1.30	0.083	0.108	0.020
15.0	9.0	6.0	12.0	0.65	0.98	1.20	0.081	0.100	0.012
12.5	5.3	7.2	11.3	0.57	0.75	1.02	0.066	0.090	—
12.5	5.3	7.2	11.3	0.80	1.07	1.15	0.095	0.102	0.019
							0.091 ± 0.004		0.013 ± 0.004
							$\sigma = 0.011$		$\sigma = 0.008$

illustrated in Fig. 3 b, and in Fig. 5 c is shown a photometer curve of the X-ray image of an extracted fiber. The beam at B travels a longer distance in the non extracted part of the myelin in proportion to the extracted part than does the beam at A. If these facts are taken into account the absorption per μ axoplasm will be about 0.025 foil equivalents, thus the same value as in the non extracted fiber. *The axon thus contains no appreciable amounts of lipids. Calculations from the values presented suggest that the middle part of the myelin sheath contains about 50 per cent lipids.*

V. DISCUSSION

All the measurements had to be performed on dried material in the high vacuum of the X-ray tube in order to minimize the absorption of the extremely soft X-rays in air. Therefore it is important to determine whether the fibers changed their form, collapsed or shrunk.

From Table I it can be seen that the nerve fibers cannot have collapsed very much. If the fiber is supposed to be an anisodiametric tube with the longest diameter parallel to the preparation holder the value of B : 2X will be greater for an elliptical than for a circular cross section using the measured values of R and r. (R-r) will have about the same or a slightly less value and therefore a negative expression is obtained for $\frac{A - \frac{B}{x}(R-r)}{2r}$ also when this expression is modified according to a non cylindrical cross section of the fiber. The X-ray pictures of the fibers, however, show that there is some amount of irregularly distributed material in the axon after drying. In the fiber (cf. Table I) with the diameters $2R = 10\ \mu$ and $2r = 5\ \mu$ the value of B : 2X will be 0.14 instead of 0.12 if the cross section is supposed to be an anisodiametric figure with the axis ratio 1.7: 1 instead of a circular. The expression $\frac{A - \frac{B}{x}(R-r)}{2r}$ then gets a negative value. The absorption value per μ myelin is thus not changed very much if the fiber changes its form a little. The values of the absorption per μ axon, however, calculated from a circular cross section might be regarded as maximal values. In Table I measurements on one fiber show no content in the axon. This might be due to either that the fiber is somewhat collapsed or that the axon has shrunk irregularly. In two microradiograms taken at an incidence of the X-rays of 45° and 90° the ratio between the fiber diameters was 1.39 (cf. fig. 4) indicating that the fibers had not collapsed very much.

Another check can be obtained from the photometer curve across the X-ray image of the fibers. Such a curve is shown in Fig. 5 a. If the X-ray absorption is calculated for different points on the fiber (cf. Fig. 3 a) the curve in Fig. 5 b is obtained for a round fiber. For a flat, collapsed, fiber the absorption curve will be flat.

The calculations are performed according to the following (cf. Fig. 3 a). μ_m and μ_a are the linear absorption coefficients for the myelin sheath and the

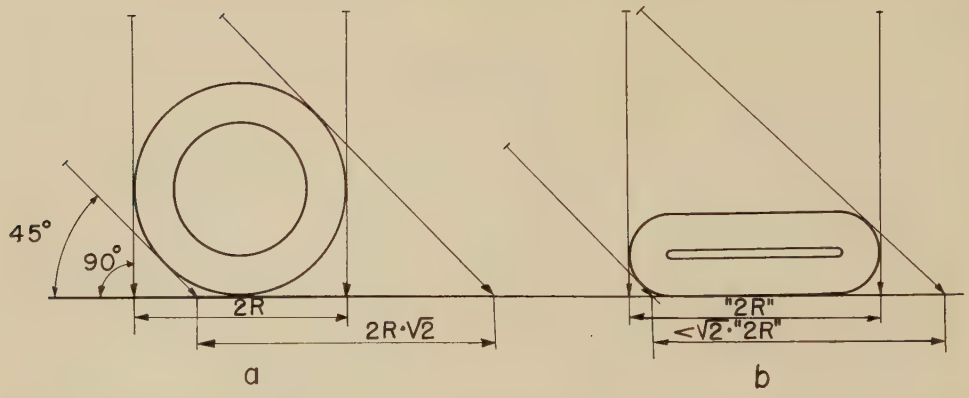


Fig. 4. a and b, the size of the microradiographic image from a round and collapsed tube when the X-rays have an incidence of 90° and 45°.

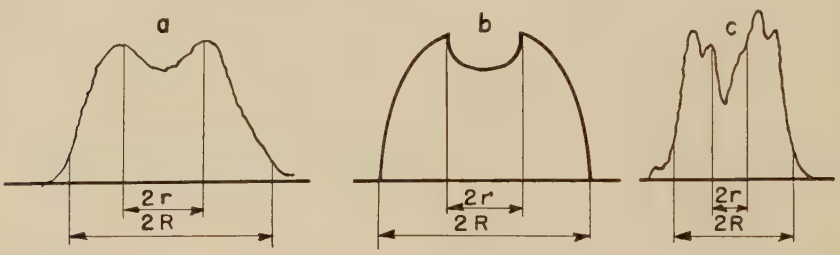


Fig. 5. a, photometer curve across the X-ray image of a single nerve fiber. b, calculated absorption curve. c, photometer curve across the image of a fiber extracted with lipid solvents. The absorption of the myelin is fractionated. 2 R and 2 r are the outer and inner diameters of the tubes respectively.

axon respectively, I_0 the intesity of the incident X-rays and I the intensity of the transmitted X-rays. The absorption of X-rays then follows the equations:

$$I = I_0 \cdot e^{-[\mu_m \cdot 2\,(\sqrt{R^2-d^2}-\sqrt{r^2-d^2}\,+\,\mu_a{}^2\sqrt{r^2-d^2}]} \quad \text{for } 0 < d < r$$

$$I = I_0 \cdot e^{-\mu_m \cdot 2\sqrt{R^2-d^2}} \quad \text{for } 0 < d < R$$

The photometer curve shown in Fig. 5 a compared with the calculated curve in Fig. 5 b lends to the conclusion that the procedure used for drying did not change the form of the fibers to any considerable extent. The composition of the myelin sheath thus can be regarded as: *weight 0,3–0.4* $\times 10^{-12}$ g/μ^3

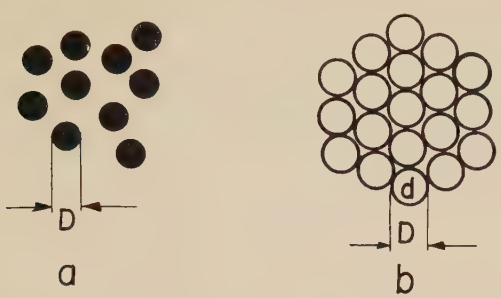


Fig. 6. Schematic representation of the axon when built up of submicroscopic fibrils of the diameter D, and when built up of tubuli of the outer and inner diameter D and d respectively.

and lipid content in the central part, 50 per cent. After the extraction with lipid solvents the photometer curve of the X-ray images aquires the shape shown in Fig. 5 c. The absorption of the myelin is fractionated.

The results presented above and obtained by absorption measurements of X-rays agree with results obtained by other techniques. A piece of frog sciatic nerve was weighed before and after drying. The dry weight was 29.5 per cent. If the individual fibers in the whole nerve have the diameters 2R : 2r as 2 : 1, three quarters of the cross sectional area lies in the myelin sheath. If the content of absorbing substances in the axon is low the approximate amount of dry substance in the myelin sheath would be about 35 per cent.

As shown in Table I the mass per unit volume of axon is very small, a maximal value of about 10 per cent dry substance is found. There are different ways in which this substance could be distributed. There could be a homogeneous solution of substance but the form birefringence of the axon (Bear, Schmitt and Young 1937) indicates the presence of elongated particles. De Robertis and Schmitt 1948, using electron microscopy found in the axoplasm fibers of indefinite length, having thin electron dense edges. They called these structural units "neurotubules". The finding by Hill and Keynes (1949) that the scattering of light in the axon increases immediately after a stimulation and is followed by a slower phase of decreased scattering of light could be explained by different aggregation of submicroscopic structural elements.

In Fig. 6 two possibilities for the structure of the axon are given. In Fig. 6 a the axon consists of fibrils with the diameter D. All fibrils, which are submicroscopic, need not be oriented in the longitudinal axis of the axon. The in situ dried axon has the approximate weight 10^{-13} g/μ^3. One fibril could

occur in a cross area with the diameter $\frac{D}{\sqrt{\rho_a}}$ where ρ_a is the average specific density of the waterfree axon and the density of the fibril is assumed to be 1. The average distance between the fibril would be about 3 to 4 D. The distance between the fibrils will be greater if the axon contains other substances in addition to them or the specific density of the fibers less than 1. The same conditions hold good for bondlike structures, a special case of Fig. 6 a.

In Fig. 6 b a close packing of submicroscopic tubes is assumed. The ratio between the two diameters of the tubes is determined by the expression $\frac{d}{D} = \sqrt{1 - \frac{27 \cdot \sqrt{3} \cdot \rho_a}{14\pi}}$ which without too big error can be simplified to $\frac{d}{D} = \sqrt{1 - \rho_a}$. Using the value 10^{-13} g/μ^3 of the axon we get $\frac{d}{D} = 0.95$. If the structural elements according to de Robertis and Schmitt l.c. have a diameter of about 600 Å. the wall thickness of the tubes would be about 15–20 Å, which is less than can be seen from their pictures. A closer packing than the one represented in Fig. 6 b, requires still thinner tubes. In this case the tubes must have irregular cross sections packed closely. As mentioned above there conceivably might be other components in the axoplasm. Therefore with the packing shown in Fig. 6 b the wall thickness would have to be less than 15 Å. A more loose packing gives a greater wall thickness.

From the experiments discussed above and which refer to myelinated nerve fibers it is not possible to decide which arrangement is the most probable. The non myelinated fibers examined by the technique described in this paper have a higher mass per μ^3 axon than the myelinated fibers. This might be due to a closer packing of the structural elements or if these are in the form of tubes that those have thicker walls.

The calculations performed above, of course, cannot give an answer to the question if the structural elements in the axon are in the form of fibrils or tubules but they suggest that the packing of the constituting elements is not very close in the myelinated fibers.

VI. SUMMARY

The determination of mass in small parts of single nerve fibers using the X-ray absorption technique described by Engström and Lindström gave the following results. The myelin sheath has the weight $0.3–0.4 \times 10^{-12}$ g/μ^3

and the axon 0.08–0.09 $\times 10^{-12}$ g/μ^3. Lipids make up about 50 per cent of the central part of the myelin sheath. No lipids could be observed in the axoplasm. The axon of non myelinated fibers seems to have a greater mass per unit volume than the axon of myelinated fibers. The ultrastructure of the axon is briefly discussed.

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A PRELIMINARY ELECTROPHORETIC ANALYSIS OF PROTEINS AND PROTEIN FRACTIONS IN SEA URCHIN EGGS AND THEIR CHANGES ON FERTILIZATION

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The large number of biochemical studies on the activation of the sea urchin egg have mainly been concerned with metabolic aspects of the problem. Little attention has been centred on changes occurring at the protein level, although profound rearrangements of the protein pattern are suggested by observations on the viscosity, permeability, cortical changes etc. occurring in the egg on activation. Mirsky's discovery (1936) that a KCl-soluble protein fraction of the unfertilized egg becomes insoluble as a result of fertilization was the first demonstration of a change occurring in a definite protein fraction.

On the other hand, even if the data so far available are too few to allow to draw any definite conclusion, it seems that differentiation in the protein pattern of the developing egg starts only rather late. The serological investigations of Cooper (1946) in Amphibia and Perlmann & Gustafson (1949) in sea urchin showing that the appearance of a new antigenic property only occurs as early as in the gastrula stage; and those of Gregg and Ballantine (quoted by Barth & Jaeger, 1947) showing the appearance of a new fibrous protein in the neurula stage, are the most indicative in this respect. During the early stages on the contrary a rearrangement of the pre-existing protein pattern takes place (see especially Örström, 1941. — For a thorough discussion on this question see Runnström, 1949). The knowledge of this process of rearrangement would be of great value, especially if ultimately one could succeed in correlating it with some cytological and physiological events.

In the spring of 1948, during a stay at the Zoological Station, Naples, Runnström investigated some of the properties of water-extracts of un-

fertilized and fertilized sea urchin eggs, especially with regard to their ability to coagulate the jelly coat in diluted sea-water. Runnström suggested to the writer to investigate the chemical nature of the active substance and its eventual changes upon fertilization. At the same time the present author had undertaken experiments to inquire into the chemical background of the changes known to occur in the lipoprotein system of the cortical layer of sea urchin eggs upon activation (Runnström, 1923–1928; Monroy & Montalenti, 1946, 1947). Fractionation of water extracts of frozen-dried sea urchin eggs was then undertaken.

In view of the basic character of the agent in sperm extracts precipitating the jelly coat (Hultin, 1947) it was natural to suspect that the active principle in the egg extracts also corresponds to a basic protein. Monroy Oddo, however, has failed so far in her attempts to extract a basic protein from *Arbacia* eggs (unpublished). The work reported below confirms this result. The attempt to isolate a lipoprotein has also been so far unsuccessful. Nevertheless it has been possible to detect in certain protein fractions some changes occurring as a result of activation, which will be referred to here.

Owing to lack of information concerning the protein fractions of sea urchin eggs and to the small amount of material available, the ordinary methods of fractionation gave at the beginning rather unsatisfactory results. Contact with several investigators of the Wenner-Gren Institute[1] led the writer to submit both total water extracts of sea urchin eggs and the fractions obtained by precipitation with Am-sulphate to an electrophoretic analysis.

As a first approach to the problem it was decided to restrict the analysis to the very early moments following fertilization. It is indeed just in the first 10 min. after fertilization that the most profound changes seem to occur in the metabolism of the egg. (Among others, the respiratory changes, Warburg, Runnström etc.; the acid formation, Runnström, 1933; the degradation of proteins, Örström, 1941, 1942; are the most significant.) Mirsky's investigations indicate that most probably the same holds good for the events involving the protein pattern. Moreover it was decided to limit this first investigation to the more readily extractable proteins, i.e. water extractable fractions.

Since, however, frozen-dried material was used in this investigation, a certain amount of salts (from the residual sea-water after centrifuging of the egg suspension [see "Experimental" part]) was dissolved in the distilled water. Chlorine determinations in the solution after extraction with distilled

[1] I wish to acknowledge especially the suggestions of Doc. G. Ehrensvärd, Dr. T. Hultin and Dr. E. Vasseur.

water and precipitation of the proteins with tungstic acid, have given values of salinity of ~0.6 per cent, calculated as NaCl. According to unpublished data of Gustafson, 29.6 per cent of the protein N comes into solution from unfertilized eggs of *Paracentrotus lividus,* extracted with a 0.9 per cent NaCl solution. This percentage comes down to 26.2 per cent in extracts of eggs 30 min. after fertilization. The same holds good for the total, water-extractable N, which decreases from 54.9 per cent to 51.8 per cent.[1]

For the present investigation, water-extracts of frozen-dried eggs of *Paracentrotus lividus* were submitted to an electrophoretic analysis. The material included unfertilized and fertilized eggs (up to 30 min. after fertilization). The material was collected at the Zoological Station, Naples, during the spring of 1948.

Most of the experiments dealt with in this paper were carried out in Na_2HPO_4–KH_2PO_4 buffer 0.05 M, $\mu = 0.1$, pH = 6.80–6.90. A few experiments were also performed on extracts of unfertilized *Paracentrotus* eggs at lower (5.77–6.14) and higher pH (8.01-8.78), in this latter case Na-Veronal-HCl was used as buffer. These experiments, however, are too few to be worth discussing here. The N-content of the extracts submitted to electrophoresis ranged between 0.868 and 1.470 mg/ml. Mobilities were read directly on the screen of the apparatus. The deviation of the mobilities from the mean value did not as a rule exceed 7 per cent. Owing to the low protein concentration, boundary anomalies were seldom observed. A total of 55 experiments were made. For further technical details, see "Experimental" part.

THE ELECTROPHORETIC PATTERN OF WATER EXTRACTS OF UNFERTILIZED AND FERTILIZED EGGS

Since nothing is known about the chemical and physico-chemical characteristics of the different protein fractions from sea urchin eggs, it is rather difficult to attempt to give any classification of them. The only criterion available at present is the precipitation with Am-sulphate which, however, cannot be taken as the only basis for classification. Thus for the present the different fractions are indicated by numbers.

All the components, both from unfertilized and fertilized eggs, migrate anodically in the pH range from 5.77 to 8.78; no component has been discovered with cathodic migration.

The electrophoretic diagram of extracts of unfertilized eggs shows the following components (Fig. 1 a).

[1] When kindly giving me the above data, Dr. T. Gustafson has pointed out that they refer to frozen-dried eggs stored for one year; secondary changes of solubility might therefore have occurred.

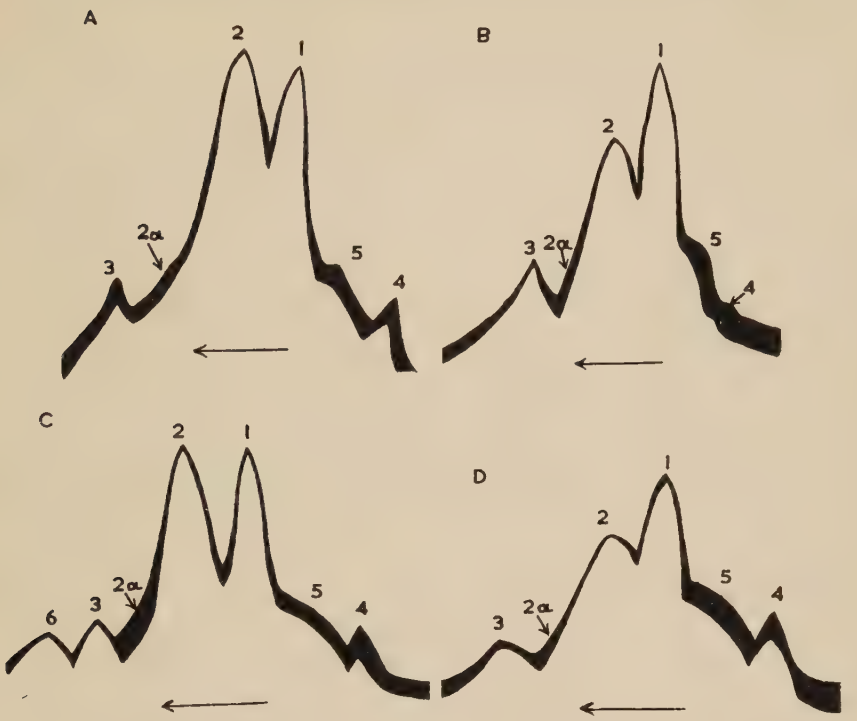

Fig. 1. *Paracentrotus lividus*. Electrophoretic diagrams of water extracts of a) unfertilized eggs; b) eggs 3 min. after fertilization; c) eggs about 10 min. after fertilization; d) eggs 30 min. after fertilization. Descending limb. pH = 6.89; Potential grad. 4.6 volt/cm. ← = direction of the migration. (All the diagrams have been prepared by tracing the enlarged image of the original photographs.)

In the central part of the diagram two big components are present: No. 1 is very sharp and keeps its sharpness throughout the run; No. 2, moving faster, undergoes blurring and, as a rule, is a little bigger than No. 1. In front of component No. 2, a small, faster moving component is present, No. 3, and behind component No. 1 a small, slowly moving one, component No. 4. Between No. 1 and No. 4 a component is present which detaches itself partially from No. 1: component No. 5. The anterior outline of No. 2 moreover shows indications of the presence of a further component, No. 2*a*; its real existence is better demonstrated by the analysis of the fractions (see later) obtained by precipitation with Am-sulphate.

In extracts of eggs 5–10 min. after fertilization (Fig. 1 c), in place of component No. 3, two components are now present, both small and similar in shape. Mobilities show that it is the slowest one which has the same mo-

Table I

Paracentrotus lividus. Total water extracts.
Mobility (u. $10^5 \cdot cm^2/volt.sec$). Descending limb.

	Components				
	1	2	3	4	5
Unfertilized eggs	3.9	5.5	8.7	1.3	—
Immed. after fertilization (3 min.) ..	4.1	6.2	8.7	—	—
5–10 min. after fertilization.....	3.6	5.2	8.8	1.6	9.7
30 min. after fertilization	3.8	5.8	8.7	—	—

bility as No. 3, whereas the faster one has a greater mobility. This latter therefore is to be considered as a new component (No. 6).

In extracts of eggs taken as soon as possible after fertilization (about 3 min., Fig. 1 b), component No. 4 is always hardly visible at the beginning of the run.

Diagrams of eggs 30 min. after fertilization (Fig. 1 d) are similar to those of unfertilized eggs, i.e. the new component No. 6 is here no longer visible.

As appears from Table I, only component No. 2 seems to undergo an increase in mobility soon after fertilization, followed by a decrease about 10 min. later. If, however, the mobilities of these components are considered after fractionation (see later) then no variation will be found. Probably therefore the variations observed in the total extracts can be due to interferences or to some other unknown reason.

ELECTROPHORETIC AND ULTRACENTRIFUGAL STUDIES ON PROTEIN FRACTIONS OF UNFERTILIZED AND FERTILIZED PARACENTROTUS EGGS

In view of the aforementioned results it was of interest to try the isolation of different components of the total water extracts and to submit them to further electrophoretic analysis. By this means it should be possible to identify some component not visible in the total diagram either because it had the same mobility as another component or because of interferences.

In order to obtain a preliminary orientation about the fractions present and the precipitations points at different concentrations of Am-sulphate, an investigation was carried out using Pedersen's method (1945).

To constant amounts of the solution to be investigated, different volumes

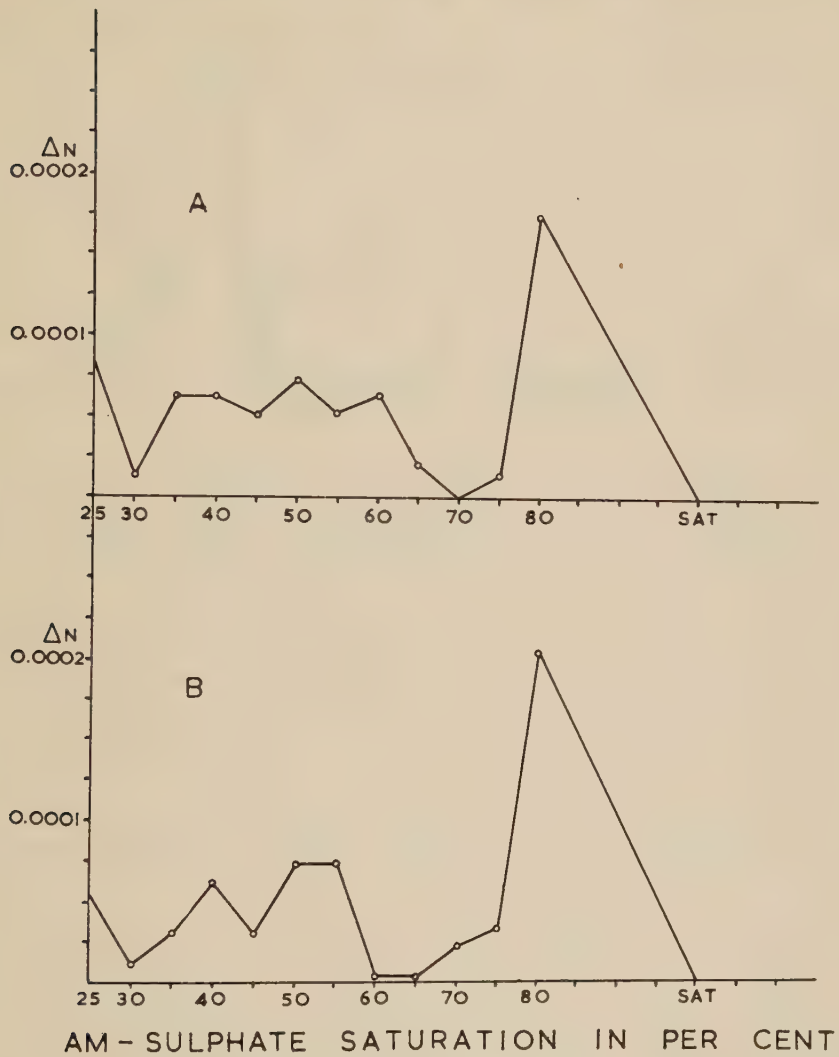

Fig. 2. *Paracentrotus lividus.* Precipitation points at different concentration of Am-sulphate of water extracts of (A) unfertilized eggs, (B) eggs 4 min. after fertilization.

of a saturated solution of Am-sulphate are added so as to get different degrees of saturation, for example from 15 up to 80 per cent. After standing overnight, the solutions are sharply centrifuged and the refractive index of the supernatant fluid (n_1) is read against the refractive index of an Am-sulphate solution at the same concentration (n_2). As long as no precipitation occurs,

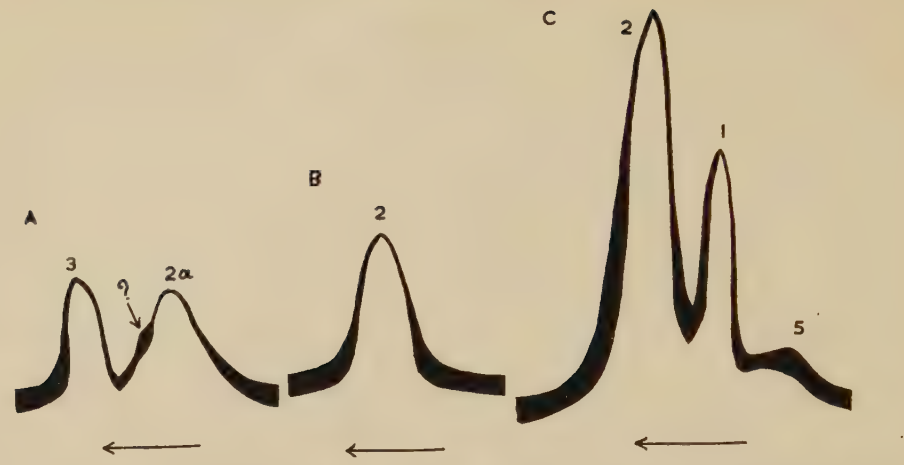

Fig. 3. *Paracentrotus lividus.* Electrophoretic diagram of the fractions precipitated from water extracts of unfertilized eggs at 30 per cent, 50 per cent and full saturation of Am-sulphate. The numbers indicate the correspondence with the components of the diagram of total extracts. ? = probable indication of the presence of a component. Exper. conditions as in Fig. 1.

the values $n_1 - n_2 = \Delta n$, will increase, but as soon as a fraction precipitates Δn will fall down, too. Plotting the Δn values against the concentrations of Am-sulphate, a graph is obtained from which a fairly good idea can be gathered of the number of fractions present in the solution and of their precipitation points.

In these experiments the concentration of proteins in the solution is rather low, and the values of Δn are therefore rather small, the differences often coming within the limits of the experimental error.

Fig. 2 a shows a diagram obtained from an extract of unfertilized eggs. It shows at least 5 precipitation points, namely one very sharp at 30 per cent, two, not very clear, between 45 and 55 per cent; one at least between 65 and 75 per cent and one about at full saturation. A diagram from newly fertilized eggs (Fig. 2 b) shows a picture which, on the whole, is similar to the one of unfertilized eggs, except that the precipitation point around 45 per cent is more clearly cut and no precipitation seems to occur at 55 per cent.

It was decided therefore to precipitate, as a first approach, at 30 per cent, 50 per cent and full saturation of Am-sulphate. For details concerning the precipitation, see "Experimental" part.

1) *Unfertilized eggs.* The fraction which is precipitated at 30 per cent saturation (Fig. 3 a) Am-sulphate shows in the electrophoretic diagram at least two components. The fastest one is very sharp and its mobility is only a

TABLE II

Paracentrotus lividus. Fractionation with Am-sulphate. Mobility (u. $10^5 \cdot$ cm.^{2}/volt.sec). Descending limb.

		Components
Fraction 30 per cent.	Unfertilized	5.1 (2a) — 8.3 (3)
	Fertilized	3.7 (2a) — 8.5 (3)
Fraction 50 per cent.	Unfertilized	5.1 (2)
	Fertilized	5.4 (2) — 9.3 (6)
Fraction sat.	Unfertilized.....	5.4 (2) — 4.3 (1)
	Fertilized.....	4.4 (1)

Numbers in brackets indicate the correspondance with the components of the total extract.

little below that of component No. 3 of the diagram of total extracts. The slowest one on the contrary, shows rather strong blurring and its mobility closely corresponds to that of component No. 2 in total extracts (Table II). It seems therefore quite reasonable to assume that this component is the component No. 2 a, which in the diagram of total extracts does not detache itself from No. 2, having the same mobility of this latter. Moreover, some asymmetries in the outline of No. 2 a suggest that it is not homogeneous. By further investigations it will perhaps be possible to isolate the different components of this fraction.

The fraction which precipitates at 50 per cent saturation of Am-sulphate appears as a single, rather small component (Fig. 3 b). Its mobility corresponds to the one of component No. 2 of the total extract diagram (Table II).

The fraction precipitating at full saturation of Am-sulphate (Fig. 3 c) contains three components. The two biggest corresponding in their shape and mobility to components No. 1 and No. 2 of the total diagram, and one similar to component No. 5. None of the three fractions obtained has shown any indication of the presence of component No. 4 of the total diagram.

2) *Fertilized eggs.* The experiments have been carried out on extracts of eggs taken between 5 and 10 min. after fertilization.

The fraction precipitating at 30 per cent saturation of Am-sulphate apparently does not undergo any variation (Fig. 4 a). The outline of the slowest component—No. 2 a—seems only to be more regular. Since, however, in these experiments the concentration of this fraction was lower than that of the corresponding one from unfertilized eggs, there is no good basis for a comparison. Considering the mobilities, a decrease of about 30 per cent of the mobility of the slowest component No. 2 a is found.

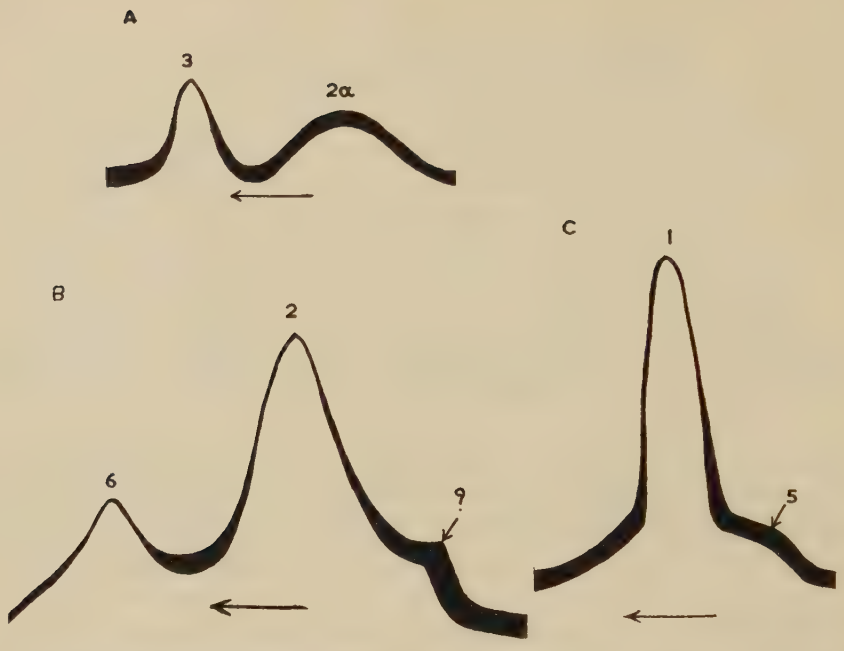

Fig. 4. *Paracentrotus lividus.* Electrophoretic diagrams of the fractions precipitated from water extracts of eggs 5 min. after fertilization at 30 per cent, 50 per cent and full saturation of Am-sulphate. Numbers and exper. conditions as in Fig. 1.

The most conspicuous changes are exhibited by the fraction which precipitates at 50 per cent saturation of Am-sulphate (Fig. 4 b). This fraction indeed now shows two distinct components, one smaller and faster and one bigger and slower. The mobility of the former corresponds to that of No. 6 and that of the latter to No. 2 and therefore also to that of the single component precipitating from unfertilized eggs at 50 per cent saturation. However, it is much bigger in size than this latter. Correspondingly at full saturation only one single main component precipitates from extracts of fertilized eggs (Fig. 4 c), namely that corresponding to No. 1, accompanied by the one corresponding to component No. 5. Therefore the component No. 2, which is mostly precipitated at concentrations of Am-sulphate above 50 per cent from extracts of unfertilized eggs, does precipitate entirely at 50 per cent saturation after fertilization. Its solubility in Am-sulphate must therefore be somewhat decreased.

One part of the fractions to be used for electrophoretic analysis was dialysed against Na_2HPO_4 0.1 M and kindly examined in the ultracentrifuge by

Dr. K. O. Pedersen, Upsala, to whom I am greatly indebted for the data which will be referred to here.

The fraction precipitated at 30 per cent sat. Am-sulphate from unfertilized eggs shows a single component with S_{20} ~8 S, whereas the same fraction from fertilized eggs shows, besides that, a large amount of very low-molecular material with S_{20} ~2 S. The 50 per cent fraction from unfertilized eggs gives a diffuse boundary with S_{20} ~2–5 S, whereas the same fraction from fertilized eggs gives a sharp component with S_{20} ~3 S. The fraction precipitating at saturation of Am-sulphate, gives in both unfertilized and fertilized egg-extracts three main components with S_{20} ~2.5–3 S; ~13–14 S; ~23–24 S.

DISCUSSION

The electrophoretic analysis of water extracts of *Paracentrotus* eggs and of their fractions obtained by precipitation with Am-sulphate, has shown a fairly complex system of components. Recently Connors & Scheer (1947) have presented an electrophoretic diagram of a KCl-extract of unfertilized eggs of *Strongylocentrotus purpuratus*. This is on the whole very similar to my diagrams of unfertilized *Paracentrotus* eggs.

The analysis of fractions obtained from *Paracentrotus* egg-extracts by precipitation with Am-sulphate has provided further evidence concerning the changes occurring at fertilization. It has been shown that the mobility of the component No. 2 a is significantly decreased after fertilization. One could assume that in extracts of unfertilized eggs component No. 2 a results in reality of component No. 2 a proper plus component No. 6. After fertilization the two components separate, component No. 6 precipitates in the 50 per cent fraction, whereas No. 2 a remains in the 30 per cent fraction and both components exhibit their own mobility. We cannot say so far whether components No. 2 a and No. 6 in unfertilized eggs keep together simply because they have the same mobility or because of the existence of a real chemical bond with each other. The decrease in mobility of component No. 2 a after fertilization seems, however, to point towards the latter possibility. The question will be submitted to further analysis.

The appearance of a large amount of low molecular material in this fraction in the ultracentrifugal diagram, could perhaps account for the far reaching degradation of proteins occurring in the egg immediately after fertilization (see especially Örström, 1941, 1942).

The decrease in solubility of component No. 2 as an effect of fertilization

seems to be also rather interesting. This component indeed whilst precipitating before fertilization very incompletely at 50 per cent of Am-sulphate, is entirely precipitated at this concentration from extracts of eggs even when taken immediately after fertilization. The result of the ultracentrifugal analysis confirms the existence of a rather profound change occurring in this fraction. No data are so far available concerning the extent of this decrease in solubility. If, however, one takes into account the diagram obtained by Pedersen's method — even considering the drawbacks due to the low concentration of our solutions — it seems that whereas in unfertilized eggs there is a fairly large and indistinct range of precipitation between 45 and 55 per cent saturation, in extracts of fertilized eggs only one and rather sharp precipitation point seems to exist, namely at 45 per cent. According to these data the decrease in solubility should be of rather small extent. One might think of it as a rather weak degree of denaturation. On the other hand the analogy seems to be suggestive between this change and the transformation Ovalbumin → Plakalbumin recently described by Linderstrøm-Lang and Ottesen (1947–1949). In this latter case the transformation occurs under the influence of an enzyme and an increase in solubility in Am-sulphate has been observed in Plakalbumin with respect to Ovalbumin. The mobility in an electric field is also somewhat changed (G. E. Perlmann, 1948). According to Runnström (1948, 1949) fertilization would involve the activation of several enzyme systems for some of which information is already available (proteolytic activity, Lundblad, 1949; alkaline phosphatase, Wicklund, 1948). It would not be surprising if a change in solubility occurred under the influence of such enzymes (see Runnström, 1949).

It is hard to say whether any connection exists between the changes described in this paper and the one observed by Mirsky. The Mirsky protein indeed is extractable in 1M KCl and becomes insoluble after fertilization. Since here we have been dealing with water extracts, it is improbable that the Mirsky protein should have come into solution. If, however, one takes into account the aforementioned data of Gustafson, then it appears that in the first 30 min. following fertilization, about 10 per cent of the water-extractable proteins become insoluble in water. This fraction could be involved in some way in the change observed by Mirsky. Lack of material has prevented fractionation of extracts of eggs 30 min. after fertilization. The diagram of the total extract, however, shows that component No. 6 is no longer visible at this time, so that these diagrams are again very similar to those of unfertilized eggs. This might perhaps be connected with the decreased solubility in water of some of the protein fractions.

EXPERIMENTAL

Preparation of the eggs. The eggs were collected by the shaking of the ovaries through Müller gauze and by washing them several times in filtered sea-water. Insemination was carried out in big bowls or, when stages had to be collected a very short time after insemination, directly in centrifuge tubes. Fertilization was controlled under the microscope and time was calculated from the moment of the first appearance of a fertilization reaction, i.e. of a slight contraction of the eggs. Batches in which the percentage of non-fertilized eggs exceeded 10 per cent were discarded. When eggs had to be collected more than 10 min. after fertilization, large rectangular glass vessels were used in which the eggs could be gently shaken in a specially constructed shaking apparatus. The egg suspensions were centrifuged as quickly as possible in a Swedish angle-centrifuge, transferred to Petri dishes, frozen at —70° C in a carbon-dioxide snow-acetone mixture and dried in high-vacuum. The dry material was finely ground and preserved in tubes in dessicators in the cold.

Preparation of the extracts. 1 g dry material was taken up in 20 ml dist. water and extracted in the cold with frequent shaking for 3 hours. The insoluble parts were removed by centrifugation at 10,000 rpm in an angle-centrifuge in the cold for 45 min. and the clear solution was dialysed for two days in the cold while being shaken against the buffer to be used for the electrophoresis. The buffer was changed twice during the first 24 hours and then the dialysis was continued against the whole of the buffer to be used in the experiment. The extracts were then centrifuged again at 10,000 rpm. For the electrophoresis, the Tiselius apparatus was used. In the first experiments the short cells were used, later on the long cells were preferred whenever possible. The temperature of the water-bath was kept constant at +1.2° C.

Fractionation with Am-sulphate. To extracts prepared from 3 g dry eggs in 50 ml dist. water, centrifuged at 10,000 rpm and dialysed for 24 hours against dist. water, Am-sulphate was added, adding the weighed amount of salt to obtain the desired concentration, through a rotating cellophane-sac, in the cold. After standing overnight, the precipitate was sharply centrifuged down, washed twice with Am-sulphate at the same concentration, dissolved in dist. water and dialysed. The insoluble parts were removed by centrifugation and the solution was precipitated again. This operation was repeated three times. Finally the dissolved precipitate was dialysed 48 hours against running tap-water (+4° C) and then against the buffer as customary and submitted to electrophoresis.

SUMMARY

Water extracts of eggs of *Paracentrotus lividus* unfertilized and a short time after fertilization, were submitted to an electrophoretic analysis.

The analysis of the total extracts shows that a new component appears soon after fertilization and disappears 30 min. later.

Further details have been gathered by the analysis of the fractions obtained

by precipitation with Am-sulphate of extracts of unfertilized and fertilized eggs. By the electrophoretic analysis of these fractions it was possible to show: 1) a decrease in solubility of one component occurring immediately after fertilization; 2) the splitting of a component of the unfertilized egg, giving rise to a new component appearing after fertilization.

Some changes in these fractions are also indicated by a preliminary analysis in the ultracentrifuge.

ACKNOWLEDGEMENTS

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ON SURFACE REACTIONS IN THE SEA URCHIN EGG

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During recent decades studies have been made of the way in which cells absorb ions and substrates. In 1933 Wilbrandt and Laszt showed that the resorption of glucose in the intestines was dependent on epithelial metabolism. That the yeast cell absorbs aneurin by a transformation in the cell's surface was shown in 1941 by Sperber. Kamen and Spiegelmann showed (1948) that yeast and other microorganisms absorb phosphate by a phosphorylation mechanism in the surface of the cell. Similar results were obtained by Rothstein and Meier (1949). Lindberg showed (1948) that the unfertilized sea urchin egg has the power to metabolize radioactive phosphate in its surface without penetration of the interior of the cell by the isotope. If, on the other hand, the fertilized egg is cultivated in sea water to which P^{32} has been added, it absorbs and metabolizes the added phosphate isotope (Villie, Lowens, and Gordon (1949); Lindberg (1948)).

MATERIAL AND METHODS

The work was carried out at the Naples Zoological Station with *Paracentrotus lividus* eggs filtered through gauze and washed three times on the centrifuge. All the experiments were made with eggs without jelly coats. When unfertilized eggs were used, acidified sea water was employed to free the eggs from their jelly coats. 0.5 ml of fertilized egg were suspended in 50 ml sea water and placed with 50 ml 0.005 N – HCl in sea water. The suspension was shaken and diluted to 500 ml with ordinary sea water and the eggs were centrifuged down and washed three times. In experiments with fertilized eggs the jelly coats were removed after fertilization.

The radioactivity was determined with a counter which gave 3×10^9, impulses/minute per mC P^{32} after precipitation of the radioactive phosphate

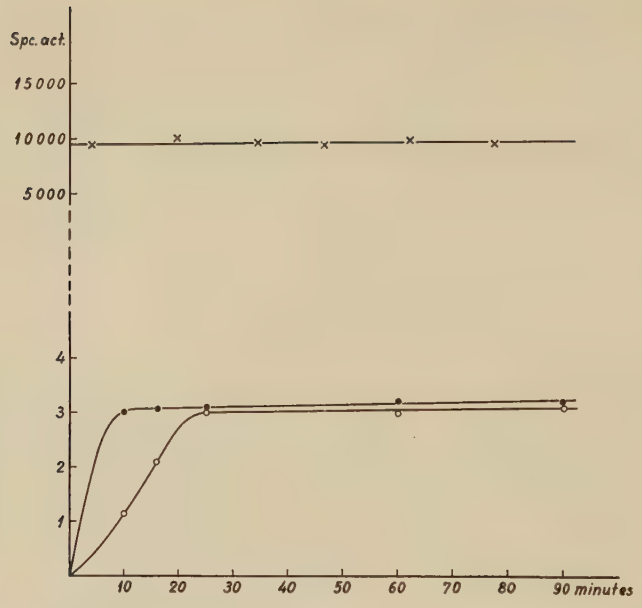

Fig. 1. Absorption and turnover of radioactive phosphate by unfertilized *Paracentrotus lividus* eggs.

●—●—●—● Specific activity in orthophosphate of eggs.
○—○—○—○ » » » ATP » »
×—×—×—× » » » orthophosphate of sea water.

as $MgNH_4PO_4$ (Lindberg 1946). The orthophosphate was determined according to Fiske and Subbarow and the ATP after 10 minutes' hydrolysis in 1 N – HCl.

EXPERIMENTS ON UNFERTILIZED PARACENTROTUS LIVIDUS EGGS

5 ml of unfertilized eggs without jelly-coats were shaken extremely carefully with 50 ml sea water containing 3.65 mg of orthophosphate and 5 μC P^{32}. After different periods of time 10 ml samples were taken and centrifuged and washed with sea water twice on the hand centrifuge. The centrifuged mass was fixed with 10 per cent TCA and the number of impulses in the ortho- and labile[1] phosphate and the corresponding quantities of phosphate were determined. The curve in Fig. 1 was obtained.

[1] Phosphate is called labile when it is hydrolyzable in 10 minutes in N–HCl at 100°. It is probably identical with ATP (adenosine triphosphate). See Zielinski (1935) and Lindberg (1943). It is calculated as "labile P".

TABLE I

Unfertilized eggs incubated 30 minutes with P^{32}. Thereafter eggs washed rapidly and number of impulses in orthophosphate and ATP determined. Reacting quantity ATP calculated from these values. All weights expressed in μg P.

Example of calculation: $\frac{1{,}220}{x_{ATP}} = \frac{430{,}000}{31}$; $x_{ATP} = 0.087$

x_{ATP} = reacting ATP in μg.

Sea water		Eggs					
Added Amount		ortho-P		ATP		Reacting Amount ATP	
ortho-P	imp/min	weight	imp/min	weight	imp/min	in μg	in per cent
31	430,000	1,210	1,400	470	1,220	0.087	0.019
62	430,000	1,280	1,100	460	610	0.087	0.019
124	430,000	1,160	905	430	405	0.117	0.027
186	430,000	1,200	795	440	170	0.074	0.017
47	1,250,000	584	7,100	154	3,040	0.11	0.07
94	1,250,000	565	5,800	145	1,260	0.097	0.06
188	1,250,000	558	4,900	140	1,030	0.15	0.10

A suspension of unfertilized eggs was mixed well and divided in 4 equal parts on 10 ml. To each part was added an equal amount of P^{32} (c. 1 μC) but increasing quantities of orthophosphate. The eggs were washed after 30 minutes and fixed as in the preceding experiment. See Table 1.

EXPERIMENTS ON FERTILIZED PARACENTROTUS LIVIDUS EGGS

1.5 ml of fertilized eggs without jelly coats (the volume was determined on an aliquot of *unfertilized* eggs) were incubated in 300 ml of sea water with 1.5 μC P^{32}. Samples of 20 ml were taken after different periods, centrifuged on the hand centrifuge and washed as above. The curve "control" in Fig. 2 was obtained for activity in orthophosphate and labile phosphate.

This experiment was repeated but after 67 minutes the suspension was divided in 4 equal parts to each of which increasing amounts of Na_2HPO_4 were added. Curves *a*, *b*, and *c* were obtained (Fig. 2). 8 μg of ortho-P per 20 ml was added to *a*, 121 μg to *b*, and 1.21 mg to *c*.

The experiments were repeated with phosphate additions between curve *a*

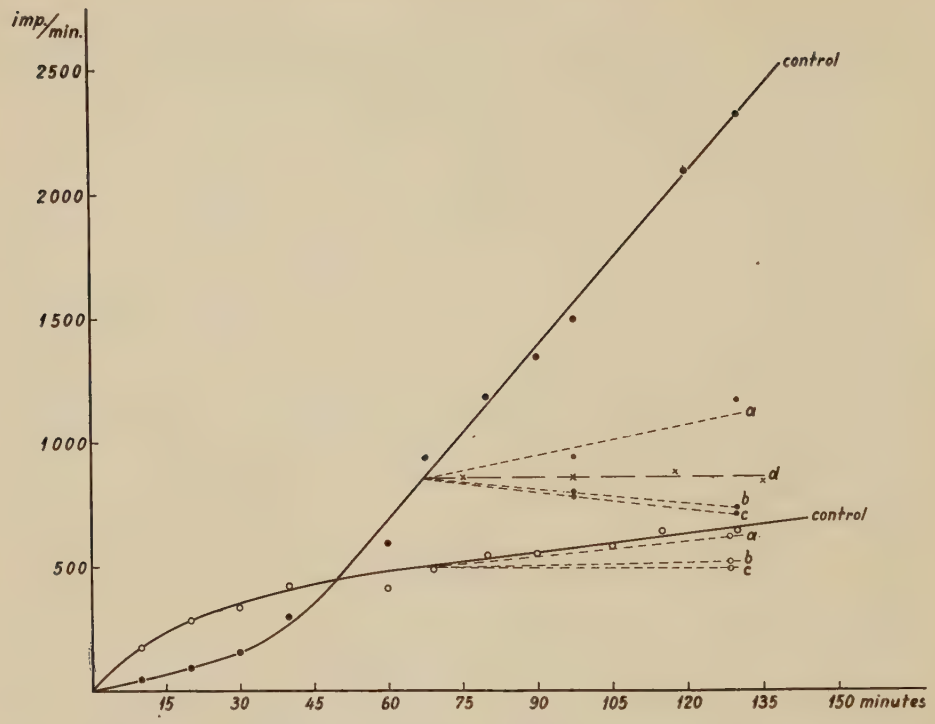

Fig. 2. Absorption and turnover of radioactive phosphate by fertilized *Paracentrotus lividus* eggs. After 67 minutes the sample was divided and different amounts of orthophosphate were added to the suspensions. Curves: Control, a, b, c, and d were obtained. ● = ATP ○ =orthophosphate × = ATP in curve d. Sample volume = 20 ml. Impulses/min. per sample 308,000.

Eggs contained 132 mg orthophosphate per sample.
» » 50 » ATP » »

0 μg	orthophosphate	per	sample	added	to	suspensions: Control
8 μg	»	»	»	»	»	a
121 μg	»	»	»	»	»	b
1.21 mg	»	»	»	»	»	c
24 μg	»	»	»	»	»	d

and curve *b*. For 24 μg P per 20 ml curve *d*, which is parallel with the abscissa, was obtained. For the sake of simplicity many curves between *a* and *b* have been excluded in Fig. 2.

Fertilized eggs were incubated with P^{32} at different times after which they were washed very carefully four times. The eggs thus *labelled* were cultivated in open rocking containers for several hours during which a number of samples were taken and the number of impulses in ortho-P and ATP determined. Fig. 3 shows the curves.

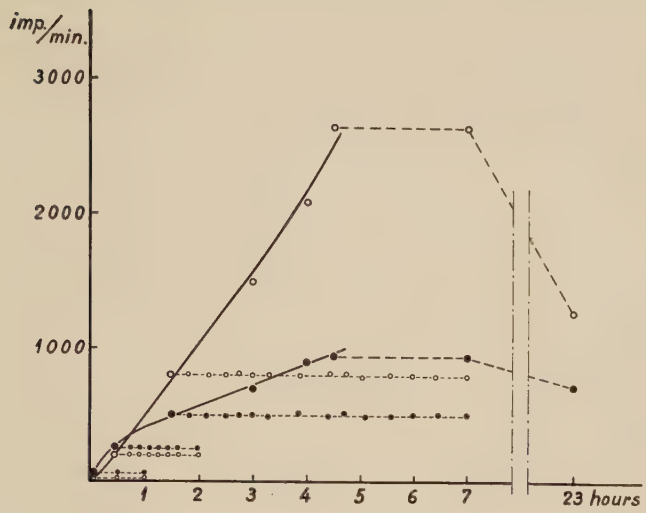

Fig. 3. Four experiments shown on one curve. Figure shows constancy in labelling amount in ATP and orthophosphate following interruption of supply of isotopes by washing the eggs. Eggs washed 5 min., 45 min., 1.5 hours, and 4.5 hours after beginning of incubation. Solid lines before washing. Dotted lines after washing. Open circles = ATP. Shaded circles = orthophosphate.

An attempt was made to determine the leakage of P^{32} from eggs previously cultivated in a radioactive medium. Fertilized eggs were shaken 90 minutes with radioactive phosphate, then washed and suspended in fresh sea water mixed with orthophosphate. 10 ml samples were taken and the number of impulses in the sea water was determined following precipitation as usual with $MgNH_4PO_4$. Wholly reproducible curves could not be obtained, but all displayed a tendency to reach equilibrium after 60–120 minutes.

In order to establish whether or not the fractions determined as "ortho-P" and "ATP" in the egg were homogeneous from a chemical or cytological point of view, we carried out the experiment reproduced in Table 2. Fertilized eggs were cultivated over different periods of time in radioactive sea water. Thereafter they were washed carefully on the hand centrifuge. The centrifuged egg mass was mixed with 5 ml saturated ammonium sulphate solution and filtered through ordinary filter paper. The precipitate was washed twice with 10 ml saturated ammonium sulphate solution. It was then impossible to extract any more phosphate by this means. Thereafter the eggs were washed with 12 ml 5 per cent TCA solution and the rest of the "TCA-soluble" phosphate was dissolved. The phosphate and specific

TABLE II

Fertilized P^{32} containing eggs extracted with saturated ammonium sulphate solution and thereafter with trichloracetic acid. Radioactivity and phosphate content determined. Table shows heterogeneity in orthophosphate and ATP.

Time of Incubation	Fractionated Extraction With				
		Saturated Am. Sulf. Solution			TCA
		1	2	3	4
90 min.	ortho-P	336	210	66	1,380
	ATP	226	165	94	400
	impulses (ortho)	3,100	2,100	740	2,500
	impulses (ATP)	1,050	—	420	5,000
	spec. act. (ortho)	9.2	10.0	11.2	1.8
	spec. act. (ATP)	4.7	—	4.4	12.5
60 min.	ortho-P	430	148	77	735
	ATP	82	52	9	365
	impulses (ortho)	20,800	8,800	3,800	21,000
	impulses (ATP)	4,500	3,300	540	39,000
	spec. act. (ortho)	48	60	49	29
	spec. act. (ATP)	56	63	60	107

activity of the extract were determined as usual. Upon analysis of phosphate fractions other than ortho- and labile phosphate, it appeared that remarkably small quantities of P^{32} were incorporated by the latter after a 2 hour incubation of the eggs in a medium containing P^{32}. See further Villie and associates (1949).

RESULTS AND DISCUSSION

Unfertilized eggs

Figure 1 shows that in a suspension of unfertilized eggs to which radioactive phosphate is added, labelled ATP is formed; it also shows that this formation reaches an asymptote after only about 30 minutes without formation of any larger amount of labelled ATP. When this asymptote has been reached there must be an exchange equilibrium between the orthophosphate and ATP in the eggs and the orthophosphate in the solution. In other words: the specific activities of the three phosphate fractions must be the same. A glance at the values in Fig. 1 indicates that under these circumstances

only a small part of the orthophosphate and ATP in the egg can have exchanged with the orthophosphate in the solution. This phosphate should be located in the surface of the egg outside an impermeable barrier.

Table 1 shows the results of attempts to regulate the eggs' absorption of isotopic phosphate by adding known amounts of orthophosphate to the suspension. Since the incubation time in these experiments was 30 minutes, there was equilibrium in the exchange between the different fractions (see Fig. 1); thus it was possible to calculate the amount of exchanging ATP (see Table 1). If we make the very rough approximation that the ATP was distributed equally throughout an egg with a diameter of 100 μ, we obtain a thickness for the "ATP-layer" of between 0.02 and 0.05 μ. Due to the rapid outdiffusion of orthophosphate during the necessary careful washing of the eggs it was impossible to undertake a similar determination of the amount of orthophosphate in the "surface" outside the phosphate-impermeable barrier.

Fertilized eggs

If, on the other hand, fertilized eggs are cultivated in a radioactive medium in the same way as described above for unfertilized eggs, no equilibrium in the exchange between the different reacting media will be reached. One obtains a constantly ascending curve for the labelling of ATP, in the course of which the specific activity of this fraction comes gradually many times to exceed that of the orthophosphate (see Fig. 2, "Control").

Since during the early larval development the concentration of the phosphate fractions remains virtually constant in the zygote and larva, the shape of the curves for the number of impulses is the same as that of the specific activity curves. It appears from the experiment shown in Fig. 3 that the ATP which has been labelled apparently does not take part in any metabolism; had it done so after a certain period equilibrium would have been reached between this phosphate ester and orthophosphate when the supply of isotopes from outside was broken off. This would mean that the egg lacked phosphate metabolism, a circumstance contraindicated both by experiments on homogenate (Lindberg and Ernster 1948) and the fact that ATP is labelled in the experiment above. Nevertheless the fact remains that whenever the supply of isotopes is interrupted there is an, at any rate apparent, equilibrium in the system, although it is not evident in analyses of the specific activity. The specific activities of ATP and orthophosphate begin to approach one another only in the swimming blastula. Presumably this may be interpreted as meaning that in the surface of the fertilized egg,

as in that of the unfertilized egg, there is a rapid metabolism which equilibrates ATP to the very high specific activity of the orthophosphate of the medium. Thence the labelled ATP penetrates slowly into the interior of the cell. This rate of transportation is the limiting factor for the labelling of ATP in the entire system.

That this transportation of the radioactive isotope takes place in the form of ATP is made probable by the fact that the specific activity of this compound increases in the course of the experiment to considerably higher values than does that of the orthophosphate. Formation of ATP from radioactive orthophosphate inside the barrier is hardly probable, since washed eggs do not equilibrate ATP with orthophosphate. Nevertheless the labelled ATP fails at any rate immediately to enter into the overall metabolism of the egg, but mingles with a relatively large amount of ATP which is separate from the rest of the ATP in the cell. That the labelling of this substance takes place primarily in only a small portion of the total quantity is also shown by the experiments in Fig. 2, in which only very small additions of ordinary phosphate are sufficient, rapidly to arrest any further esterification of P^{32}. If the same calculation of the amount of reacting ATP from the position of equilibrium is applied here as in the experiment on unfertilized eggs (in Table 1), the result is obtained that c. 0.1 per cent of the amount of ATP is in equilibrium with the outer solution. If, however, we put labelled and washed eggs into fresh sea-water we are able to follow the slow and small leakage of P^{32} from the egg into the water. If on the basis of these values we do the same calculation of the reacting amount of ATP as before results are obtained which vary considerably from one experiment to the other. About 50 per cent of the quantity of ATP is, however, a common value.

Only in the swimming blastula the equilibration between ATP and orthophosphate is approached. It might be that the energy is stored in the early larval development as ATP which in the swimming blastula is used for synthetical purpose.

Experiments with extraction of the labelled eggs with ammonium sulphate solution and thereafter TCA show, like the experiments of Kamen and Spiegelmann (1948) on yeast and other microorganisms, that orthophosphate and labile phosphate are not homogeneous fractions. Since there is neither creatine or arginine phosphate nor substances with a corresponding degree of hydrolysis in the egg (Zielinski (1935) and Lindberg (1943)), it is very probable that these differences in specific activity obtained as a result of different kinds of extraction are attributable rather to a fixation to the cell structure than to chemical differences in the ordinary sense of the word

(easily hydrolyzable organic phosphate esters). Most striking is the *large* number of impulses found in the non-ammonium sulphate extractable ATP and the small number found in the corresponding orthophosphate fraction. The question is whether this ATP should not be regarded as bound to the structure of the egg. If so, the most conceivable hypothesis is that it is the cortex which contains this large amount of very radioactive structure-bound ATP. That *protein-bound* phosphate can take part directly in the metabolism of the trichloracetic acid-soluble phosphate esters and thus absorb considerable radioactivity has been well demonstrated by Jagannathans experiments on phosphoglucomutase (1949).

The fact that the "orthophosphate" can be divided into two fractions, one of which has considerably lower specific activity than both ATP and the other fraction, can be interpreted as indicating that in addition to ordinary orthophosphate, it is made up of a component which in part at any rate is so located in the egg that the penetrating marked phosphate reaches it only slowly. The fact that the "orthophosphate" can be divided up in this way into two components one of which is extractable with ammonium sulphate solution while the other remains insoluble until the eggs are treated with trichloracetic acid suggests that the second fraction may be bound to the cell protein.

The fact that addition of relatively insignificant amounts of orthophosphate to the radioactive sea water prevents any further absorption of isotopic phosphate explains why earlier (Lindberg 1946) it has not been possible to demonstrate any absorption of P^{32} by fertilized sea urchin eggs. The isotope used then was made by the cyclotron and far too contaminated by P^{31}.

SUMMARY

The unfertilized *Paracentrotus lividus* egg is impermeable to orthophosphate, while it has the capacity to metabolize radioactive orthophosphate in its surface to adenosine triphosphate. This metabolizing layer is c. 0.02–0.05 μ thick.

If the fertilized *Paracentrotus lividus* egg is cultivated in sea water to which radioactive phosphate has been added, it takes up the isotope primarily in the form of ATP.

The fertilized egg metabolizes radioactive phosphate in the surface very rapidly. This process forms radioactive ATP which slowly makes its way into the egg.

The penetrating ATP is largely bound to the structure and plays no noteworthy part in the metabolism of the egg.

The phosphate fractions in the egg which upon analysis are called "orthophosphate" and ATP are not homogeneous. This heterogeneity is probably cytochemical in nature.

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ELEKTRONENOPTISCHE UNTERSUCHUNGEN DER STRUKTURVERÄNDERUNG AGGLUTINIERTER FISCHSPERMIEN

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EINLEITUNG

Seit den Untersuchungen Lillies (1913) über Befruchtungsstoffe der Seeigel, die ergaben, dass ein im Ei enthaltener Stoff, das Fertilizin, die Agglutination der Spermien bewirkt, wurde dieser Effekt bei einer Reihe weiterer Wirbelloser nachgewiesen. In neuerer Zeit hat M. Hartmann (1944) erstmals auch eine Spermaagglutination bei Fischen (*Salmo irideus*) festgestellt (s. Übersichtsreferat Bielig und Medem, 1949). Verschiedene Autoren, besonders A. Tyler (1948), weisen auf die Ähnlichkeit dieser Erscheinung mit Antigen-Antikörper Reaktionen hin. Die elektronenoptischen Untersuchungen von Mudd und Anderson (1941) ergaben, dass begeisselte Bakterien unter der Einwirkung von Antisera einen beträchtlichen Strukturwandel erleiden (s.w.u.). An agglutinierten Spermien wurden unseres Wissens solche Beobachtungen bisher nicht gemacht. Lediglich auf Grund seiner lichtmikroskopischen Befunde beschreibt Popa (1927) Veränderungen an den Köpfen agglutinierter Seeigelspermien.

Im Verlauf einer Untersuchung über Ei- und Spermawirkstoffe (Gamone) verschiedener Teleosteer (Medem, Rötheli und Roth, 1949) gelang es uns mit Hilfe des Elektronenmikroskopes nachzuweisen, dass die Spermien beim Agglutinationsvorgang tatsächlich morphologisch erkennbare Veränderungen erfahren, die in mancher Hinsicht denjenigen von Bakterien recht ähnlich sind. In der vorliegenden Arbeit werden diese Befunde zusammen mit einigen Beobachtungen über die Struktur der Fischspermien dargestellt.

MATERIAL UND METHODE

Zur vergleichenden Untersuchung der Agglutination wurden die Spermatozoen folgender Teleosteer verwendet:

A. *Esocidae* 1) *Esox lucius* L. Hecht aus dem Wohlen- und Bielersee.

B. *Salmonidae* 1) *Salmo trutta* L. Flussforelle aus der Aare bei Bern.

2) *Thymallus vulgaris* Nils. Äsche aus der Aare bei Bern.

3) *Coregonus* L. Felchen.

a) *C. schinzii* Fat. „Balchen" des Thunersees.

b) *C. wartmanni* Bloch „Pfärrit" des Bielersees.

c) *C. wartmanni* Bloch „Schwebfelchen" des Zürichsees.

d) *C. asperi* Fat. „Albeli" des Zürichsees.

Die Geschlechtsprodukte wurden nach der von uns (1949) beschriebenen Methode gewonnen. Für die elektronenoptische Untersuchung brachte man eine möglichst geringe Anzahl der Spermatozoen in das zu prüfende Medium und übertrug danach einen Tropfen dieser Suspension mit Hilfe einer feinen Pipette auf die Zellulose-Trägerfolie.[1] Nach dem Eintrocknen wurden die Präparate direkt oder nach erfolgter Goldbeschattung (Beschattungswinkel $\operatorname{tg} \alpha = 1/4$) untersucht. In einigen Fällen fixierten wir voll aktive oder bereits bewegungslose Spermien mit *Zenkerscher* Flüssigkeit.

DIE STRUKTUR NICHT AGGLUTINIERTER SPERMIEN

Im Spermaliquor sind die Spermien unbeweglich. In Wasser gebracht, beginnen sie sich sogleich lebhaft zu bewegen. Nach kurzer Zeit wird die Bewegung langsamer, dann zitternd und hört endlich ganz auf. Art und Dauer dieses Bewegungsablaufes sind für jede der untersuchten Fischarten charakteristisch (Medem, Rötheli und Roth, 1949).

Die elektronenoptische Untersuchung unfixierter Spermien erfolgte stets nach Schluss des normalen Bewegungsablaufes. Köpfe und Schwänze sind immer *scharf konturiert*, nie scheinen sie im Wasser stark aufgequollen oder zeigen eine durchscheinende, verschwommene Randzone. Meist ist

[1] Zur Verfügung stand uns das Trüb-Täuber Elektronenmikroskop der Universität Bern. Die Aufnahmen wurden durch Zuwendungen aus den Arbeitsbeschaffungskrediten des Bundes ermöglicht. Herrn Dr. H. Studer, Chemisches Institut, möchten wir an dieser Stelle für seine wertvolle Mitarbeit herzlich danken.

Tab. I.

Vergleichende Messungen an Fischspermien.
Masse in μ.

Art	Kopf			Schwanz			Fibrillen		
	Breite	Länge	Dicke	Breite	Länge	Dicke	Anzahl	Breite	Dicke
Esox lucius	2	2	0,3	0,21	35 40	0,01	11	0,035	0,008
Esox Zenker fix	1,5–2	1,5–2	1,5	0,14	35–40	0,08	—	—	—
C. wartmanni (Biel)	2	3	—	0,16	40	—	ca 9	0,035	—
C. wartmanni (Zürich)	2,75	3,25	—	0,17	40–45	—	—	—	—
C. asperi	3	3,5	—	0,15	40	—	—	0,035	—

der Schwanz mehr oder weniger gerade gestreckt, seltener liegt er in losen Schlingen um den Kopf (vgl. die Beobachtungen von Harvey und Anderson an Seeigelsperma, 1943). Stellenweise, besonders aber am Ende, ist er *aufgesplittert* und lässt deutlich seinen fibrillären Aufbau erkennen (Abb. 1 d, 2, 4). Alle Fibrillen eines Spermiums sind unter sich und auf der ganzen Länge gleich dick. Die Masse von Kopf, Schwanz und Fibrillen sind in Tabelle 1 dargestellt. Ihre Querschnitte wurden beim Hechtsperma mit Hilfe der Goldbeschattung ermittelt; sie erwiesen sich als oval. Diese abgeflachten Formen sind vermutlich eine Folge des Eintrocknens (s.w.u.).

Allgemein zeigt sich, dass die Breite der Elementarfibrillen aller hier untersuchten Fischspermien übereinstimmt. Die genaue Anzahl wurde nur beim Hecht (11 Fibrillen) ermittelt (Abb. 1 d, 2). Die Form des Kopfes ist bei den einzelnen Arten verschieden. Beim Hecht ist sie nahezu kreisrund (vgl. a. Ballowitz, 1890), bei *Coregonus wartmanni* (Bielersee) eichelförmig (Abb. 4). *Coregonus wartmanni* (Zürichsee), *Cor. asperi* und *Salmo trutta* haben länglich-kirschförmige Köpfe. Ein Verbindungsstück, wie es Ballowitz (l. c.) beschreibt, haben wir nur in seltenen Fällen an fixiertem Material feststellen können. Hingegen scheinen beim Hecht (Abb. 4 a) Verbindungsfasern vorhanden zu sein, wie sie Bretschneider und van Iterson (1947) beim Stiersperma als „articular strands" beschrieben haben.

Vergleicht man die fixierten Spermien mit dem unfixierten Material (vgl. Tab. 1; *Esox lucius*), so fällt auf, dass die Köpfe und Schwänze weniger abgeflacht sind. So erscheint z. B. der Kopf des Hechtspermiums nahezu kugelförmig, was seiner wirklichen Gestalt am nächsten kommen dürfte.

An Hechtspermien, die sofort nach dem Einbringen in Wasser fixiert wurden, erkennt man deutlich einen Plasmasaum, der sich längs des Schwan-

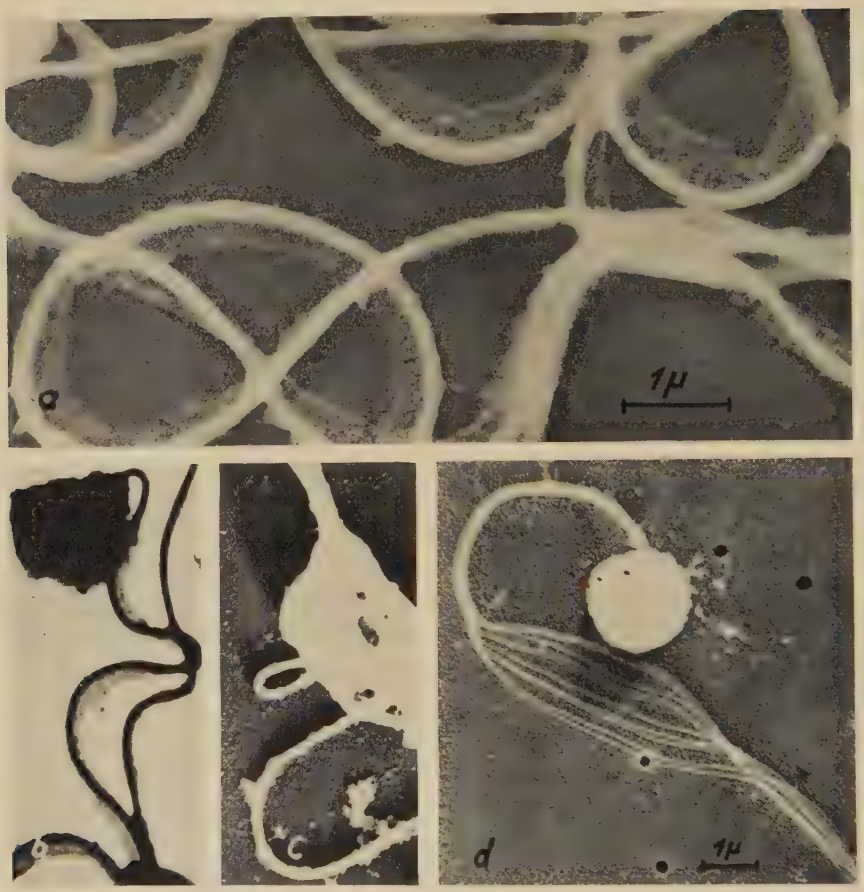


Abb. 1.
Hechtsperma (*Esox lucius* L.).

a) Sperma unmittelbar nach dem Einbringen in Wasser fixiert (Zenker), goldbeschattet. Schwanz nicht aufgesplittert. Plasmasaum.
b) Wie Abb. a aber nicht goldbeschattet.
c) Sperma 4 min nach Einbringen in Wasser fixiert (Zenker), goldbeschattet. Der Plasmasaum ist verschwunden.
d) Sperma in Wasser präpariert, goldbeschattet. Der Schwanz lässt deutlich seinen Aufbau aus 11 Fibrillen erkennen.

zes bis gegen das Ende hinzieht (vgl. Ballowitz, 1890; Retzius, 1905). Nach unseren Befunden ist dieser Saum bis 0,3 μ breit, ca. 0,007 μ dick und von faseriger, gewebeartiger Struktur. Ob er dem Schwanz nur einseitig anliegt, oder diesen spiralig umwindet, konnte bisher nicht eindeutig festgestellt wer-

Abb. 2.

Esox lucius Einzelspermium, Totalansicht.

Aus 5 Aufnahmen zusammengesetzt. Schwanz in einzelne Fibrillen aufgesplittert, Plasmasaum aufgelöst. Die scheinbare Kordelstruktur der Fibrillen ist durch den Raster verursacht.

den. Da die Spermien im Zeitpunkt der Fixierung noch voll aktiv waren, sind die Schwänze nie gerade gestreckt, sondern meist unregelmässig gebogen oder gewellt. Der Plasmasaum liegt stets an der Innenseite des Bogens (Abb. 1 a, b), und zwar ist er um so breiter, je stärker dessen Krümmung erscheint. Sein Rand ist leicht verdickt. Dieser Saum kann sich also offenbar streckenweise verkürzen, und wir sind im Gegensatz zu Ballowitz (l.c.) der Ansicht, dass er ein kontraktiles Element darstellt.[1] Möglicherweise wirkt das Fibrillenbündel des Achsenfadens nach Art einer elastischen Chorda der Verbiegung durch den Plasmasaum entgegen. Tatsächlich ist an geraden Schwanzstücken der hier gestreckte Saum meist überhaupt

[1] Die gleiche Funktion besitzt nach Terni (1933) die undulierende Membran am Spermaschwanz von *Geotriton fuscus* und *Triton alpestris*. Sie soll auf Grund ihres fibrillären Aufbaues den ausschliesslichen Sitz der Bewegung darstellen.

nicht, oder lediglich als sehr schmale Randzone zu erkennen (Abb. 1 a). Für diese Annahme sprechen auch die Befunde an Spermien, die erst 4 min nach dem Einbringen in Wasser also in vollständig bewegungslosem Zustand fixiert wurden. Die Schwänze sind jetzt meist gestreckt oder bilden nur sehr wenig gekrümmte Bogen. Der Plasmasaum ist weitgehend aufgelöst, selten erkennt man einige seiner Reste (Abb. 1 c). Das Verschwinden dieses Saumes scheint mit der Abnahme der Beweglichkeit parallel zu gehen. Auch Ballowitz (l.c.) beschreibt die ausserordentliche Zartheit dieser Bildung: Sie wird in schwachen Kochsalzlösungen — nach unseren Befunden auch in Wasser — innerhalb kurzer Zeit aufgelöst, und der Schwanz zerfällt in einzelne Fibrillen.

DIE STRUKTURVERÄNDERUNGEN AGGLUTINIERTER SPERMIEN

Mit Wasser verdünnter Eiinhalt bringt arteigene Spermien der von uns untersuchten Fische zur Agglutination (*Isoagglutination*). In einigen Fällen wird auch artfremdes Sperma agglutiniert (*Heteroagglutination*). Bei der Äsche übt ferner verdünntes Fruchtwasser die gleiche Wirkung aus (nach Hartmann, 1944, Hartmann, Medem, Kuhn und Bielig, 1947, ist das auch bei der Regenbogenforelle der Fall). Der Agglutinationsvorgang spielt sich folgendermassen ab: Wird voll aktives[1] Sperma in das agglutinierende Medium gebracht, so beginnt es sofort oder nach einigen sec mit der Bildung kleinerer Zusammenlagerungen. Anschliessend vergrössern sich diese, treten miteinander in Verbindung und bilden weitmaschige Netze, in denen die Spermien mit Köpfen und Schwänzen verklebt sind (vgl. Abb. 3 b). Es handelt sich also stets um eine irreversible Kopf- und Schwanzagglutination. Die Spermien befinden sich noch in lebhaft zitternder Bewegung. Nach ihrem Absterben beginnen die Netze zu reissen und schrumpfen zu gröberen Anhäufungen zusammen (Abb. 3 c). Zwischen Iso- und Heteroagglutination fanden wir in dieser Hinsicht keinen Unterschied. Im Gegensatz dazu stellt u.a. auch Vasseur (1947) auf Grund seiner Untersuchungen an Seeigeln fest, dass die Isoagglutination in erster Linie eine Kopf-, die Heteroagglutination dagegen eine Schwanzagglutination darstellt.

Agglutinierendes Sperma zeigt gegenüber solchem in Wasser durchgehend die gleichen Veränderungen. Die einzelnen Spermatozoen sind fast ausschliesslich zu grösseren Verbänden vereinigt. In diesen Zusammenballun-

[1] Unbewegliche Spermien konnten nie zur Agglutination gebracht werden. Vasseur (1949) fand das Gleiche bei Spermatozoen verschiedener Seeigelarten.

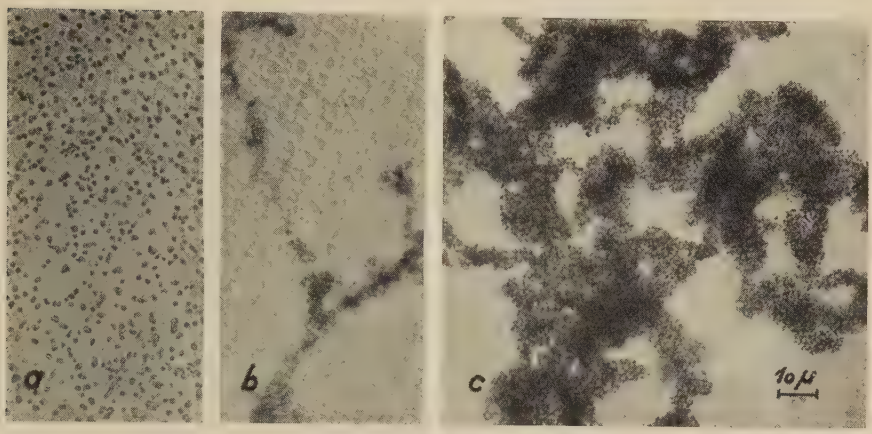


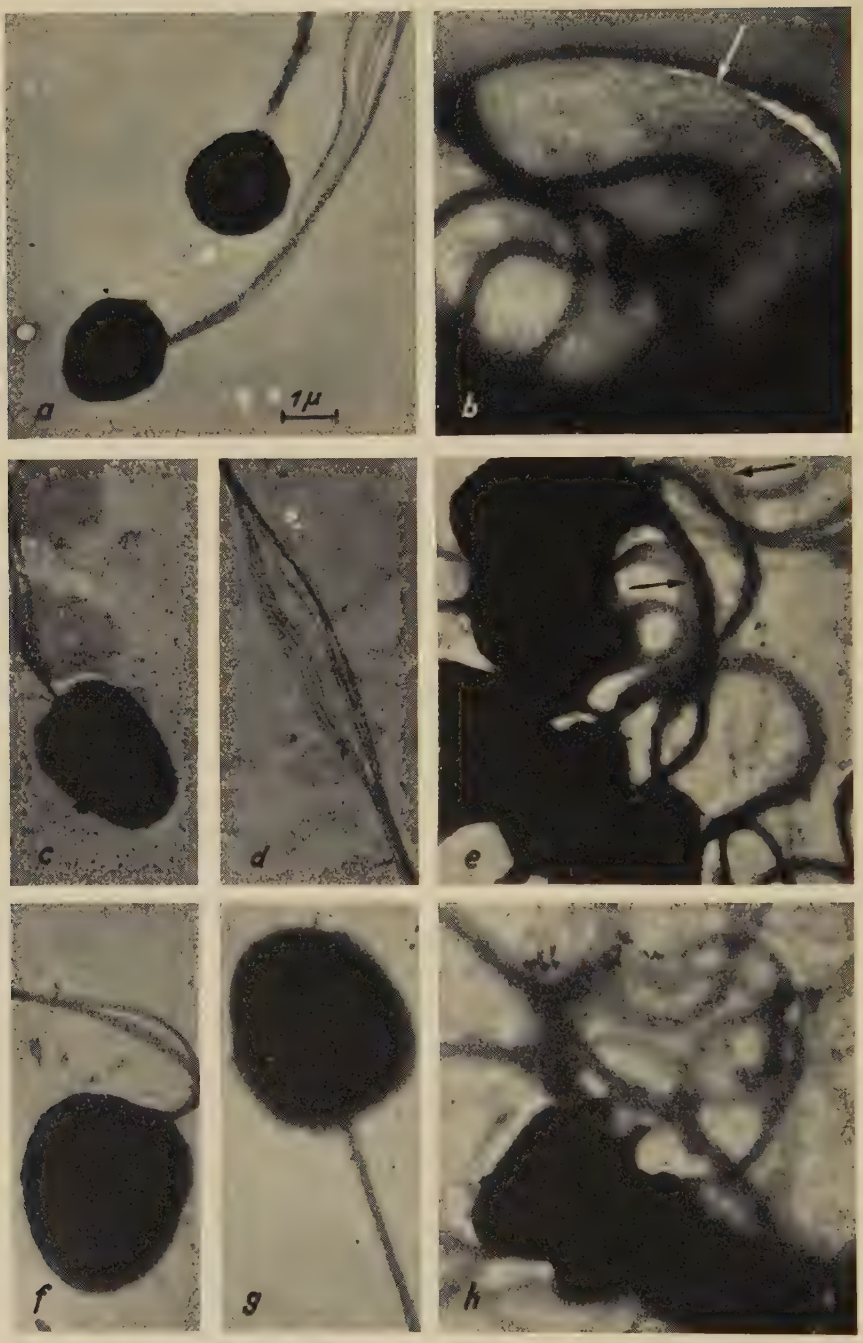
Abb. 3.

Isoagglutination von Salmoniden-Sperma.

a) Normale Verteilung von Felchensperma in Wasser. (*Cor. wartmanni*, „Pfärrit".)
b) Agglutination. Beginn der Netzbildung. (*Salmo trutta*, Flussforelle: 5 vol % Eiinhalt + 95 vol % Wasser.)
c) Endphase der Agglutination. (*Cor. wartmanni*, „Pfärrit": 2 vol % Eiinhalt + 98 vol % Wasser.)

gen berühren sich die Köpfe gegenseitig und sind oft so stark verklebt, dass sie nicht mehr voneinander abgegrenzt werden können (Abb. 4 b, e, h). Meist sind sie dabei nicht grösser als die Spermienköpfe in Wasser, nur einzelne zeigen eine starke Quellung. Die Schwänze sind nicht, wie bei Spermien in Wasser, mehr oder weniger gestreckt, sondern bilden ein engmaschiges Geflecht. Ihre Konturen sind verwischt; stellenweise zeigen die Schwänze sogar eine weitgehende Auflösung. Ähnliche Erscheinungen beobachteten Mudd und Anderson (1941) an agglutinierten Bakterien: Die Zellwände waren durchscheinend und verschwommen, die Geisseln verdickt (z.T. durch Auflagerung eines Antikörper-Filmes) und weniger scharf begrenzt. Einzelne Elementarfibrillen konnten an den Spermaschwänzen nicht mehr festgestellt werden. Dagegen fanden wir hin und wieder (Abb. 4 e) an ihrer Stelle sehr feine, für Elektronen weitgehend undurchlässige, nadelartige Stäbchen. Sie sind durchschnittlich 0,001 µ breit, 0,03–0,3 µ lang und stets in der Längsrichtung des Schwanzes angeordnet.

Beim Hecht kann man besonders deutlich die scharfe Grenze einer schleimigen Zone erkennen, innerhalb der die Köpfe und Schwänze zu einer Masse verklebt sind (Abb. 4 b). Nach den Befunden Popas (1927) bildet sich unter dem Einfluss von Eisekretwasser des Seeigels *Arbacia punctulata*


a
1μ
b
c
d
e
f
g
h

bei arteigenen Spermien an der Spitze des Kopfes eine Mikropyle. Durch diese Öffnung tritt eine Substanz aus, die das Verkleben der Spermienköpfe untereinander bewirkt. In diesem Stadium ist die Agglutination noch reversibel, und die Spermatozoen sind in Bewegung. Später löst sich die plasmatische, lipophile Hülle, die den Kern umgibt, allmählich ab und häuft sich als „Lateralkörper" aussen am Kopf des Restspermiums an. Die Agglutination ist jetzt irreversibel, und die Spermien werden unbeweglich. Wir konnten nicht feststellen, ob bei agglutinierten Fischspermien dieser Schleim wie bei Seeigeln, nur von den Köpfen herstammt, doch vermuten wir, dass diese Substanz auch von den Schwänzen herrührt.

Die Intensität der Zusammenballung ist von der Konzentration des agglutinierenden Agens abhängig. In Anlehnung an Hartmann et al. (1947), haben wir die Agglutination in Stärkegrade I–IV eingeteilt (s. Tab. 2).

Aus Tab. 2 geht hervor, dass bei Forelle und Äsche die maximale Agglutinationsstärke durch eine Konzentration von 50 vol % Eiinhalt erreicht wird. Mit steigender Verdünnung des agglutinierenden Mediums nimmt sie kontinuierlich ab. Im Gegensatz dazu findet bei Felchen und Hecht eine Agglutination nur in einem eng begrenzten Konzentrationsbereich statt. Innerhalb dieses Bereiches wird die maximale Agglutinationsstärke nur bei einer bestimmten Konzentration erreicht. Die Beobachtung ergab, dass hier vorübergehend eine besonders intensive Trübung des Mediums eintrat, was auf eine erhöhte Ausfällung der Eiprotein-Komponente schliessen lässt.

Die Heteroagglutination zeigt, dass der „Agglutinations-Typus" nicht immer vom Medium allein bestimmt wird (wie z.B. bei Hecht und Äsche), sondern auch weitgehend von der Reaktionsweise des Spermas ab-

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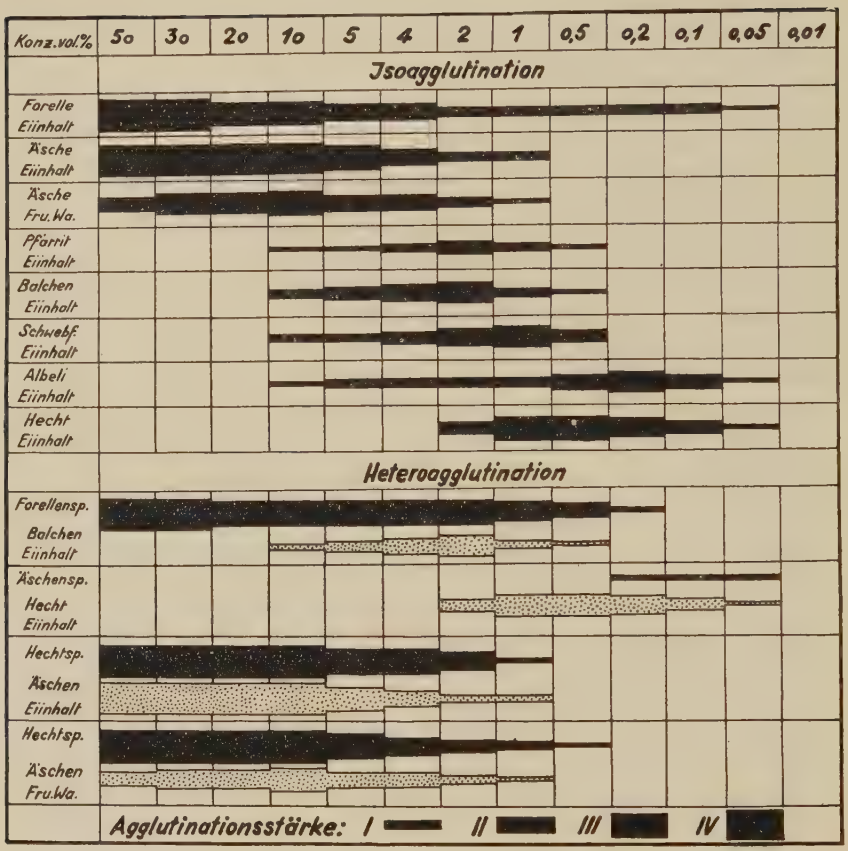
Abb. 4.

a) *Esox lucius*, Sperma in Wasser, unbeschattet. Schwanz z. T. in Fibrillen aufgesplittert. Köpfe scharf konturiert, am oberen sind Verbindungsfasern zum Schwanz zu erkennen.
b) *Esox lucius*, agglutiniertes Sperma, goldbeschattet. (0,5 vol % Eiinhalt + 99,5 vol % Wasser.) Köpfe und Schwänze unscharf konturiert und innerhalb einer schleimigen Zone verklebt, deren Grenze durch den Pfeil angegeben wird.
c & d) *Cor. wartmanni* (Biel), Sperma in Wasser, unbeschattet. Scharf begrenzter eichelförmiger Kopf (c) und aufgesplittertes Schwanzstück (d).
e) *Cor. wartmanni* (Biel), agglutiniertes Sperma, unbeschattet. (2 vol % Eiinhalt + 98 vol % Wasser.) Köpfe zu einer Masse verklebt. Schwänze stellenweise aufgelöst; hier sind in der Längsrichtung verlaufende dünne Stäbchen zu erkennen (Pfeile).
f) *Cor. asperi*, Sperma in Wasser, unbeschattet. Kirschförmiger Kopf.
g) *Cor. wartmanni* (Zürich), Sperma in Wasser, unbeschattet. Kirschförmiger Kopf.
h) *Cor. wartmanni* (Zürich), agglutiniertes Sperma, unbeschattet. (2 vol % Eiinhalt + 98 vol % Wasser.) Köpfe und Schwänze miteinander verklebt. Die letzteren grösstenteils aufgelöst.

Tab. II.



Die Abhängigkeit der Agglutination von der Konzentration des Eiinhaltes.

Die vol % geben die Verdünnung des Eiinhaltes mit Wasser an. Die Agglutinationsgrade bedeuten: I) Schwache Agglutination; keine Netzbildung, Stäbchen und kugelige Konglomerate. II) Mittelstarke Agglutination; Bildung dünner Netze nach einigen sec, später zunehmende Verdichtung. III) Starke Agglutination; sofortige Ausbildung dicker Netze. IV) Stärkste Agglutination; sofortige Zusammenballung des Spermas zu grossen Klumpen.

Die *schwarzen* Balken geben die tatsächliche Agglutinationsstärke an. Die *punktierten* stellen den Agglutinationstypus dar, wie er für das betreffende Medium bei Isoagglutination zu erwarten wäre.

hängig sein kann (z.B. Forellensperma in Balchen-Eiinhalt). Arteigene Spermien sind bei starken Konzentrationen des Eiinhaltes oft weitgehend unbeweglich, während artfremde eine unverminderte Beweglichkeit aufweisen und dann stark agglutinieren.

Mit den chemischen Grundlagen der Spermaagglutination setzt sich Vasseur (1949) auseinander. Er weist nach, dass in den Gallerte-Hüllen der Seeigeleier zwei Hauptkomponenten enthalten sind, nämlich Kohlehydrate und Proteine. Das Protein diffundiert durch die Eigallerte hindurch und wird hier im Moment der Auflösung im Wasser von Polysaccharid-Molekülen umgeben, die mit der Protein-Komponente einen festen Komplex bilden. Diese Komplexe sind das Fertilizin des Eisekretwassers, welches arteigenes Sperma agglutiniert. Die agglutinierende Wirkung wird durch Zugabe einer isotonischen Calcium-Lösung wesentlich erhöht. Dieser Effekt scheint hauptsächlich darauf zu beruhen, dass die Ca-Jonen eine Bindung des Fertilizin-Komplexes an die Spermienoberfläche erleichtern. Die Spermien wiederum enthalten Enzyme, die einen Abbau der agglutinierenden Substanz bewirken können und die sich wahrscheinlich nur in den Spermaköpfen, nicht aber in den Schwänzen befinden. Die von uns beobachtete, relativ rasch erfolgende Strukturveränderung von agglutiniertem Fischsperma könnte auf fermentativem Wege erfolgt sein, kann aber ebenso gut andere physico-chemische Vorgänge als Ursache haben.

ZUSAMMENFASSUNG

1) Es werden elektronenoptische Untersuchungen über die Spermaagglutination von *Esox lucius, Thymallus vulgaris, Salmo trutta* und von 4 *Coregonus*-Rassen angestellt. Die Struktur von nicht agglutinierten Spermien wird an Hand von fixiertem und unfixiertem Material mit derjenigen agglutinierter Spermatozoen verglichen.
2) Nicht agglutiniertes Sperma in Wasser zeigt deutlich den fibrillären Aufbau des Schwanzes (Hecht 11 Fibrillen, Felchen ca 9). Vergleichende Masse für Kopf, Schwanz und Elementarfibrillen der Spermatozoen werden angegeben.
3) Der Schwanz des Hechtspermiums besitzt einen Plasmasaum. Dieser Saum stellt wahrscheinlich das kontraktile Element dar.
4) Das agglutinierte Sperma der untersuchten Teleosteer zeigt durchwegs die gleichen Strukturveränderungen: Die Köpfe sind zu einer einheitlichen Masse verklebt, die Schwänze zu einem dichten Geflecht verstrickt und oft innerhalb einer schleimigen Zone weitgehend aufgelöst. Einzelne Fibrillen sind nicht mehr festzustellen. Diese Zusammenballungen zeigen eine grosse Ähnlichkeit mit agglutinierten Bakterien.

SUMMARY

1. The agglutination of the spermatozoa of Esox lucius, Thymallus vulgaris, Salmo trutta and of 4 races of Coregonus was observed by means of the electron microscope. The structure of agglutinated and non-agglutinated spermatozoa was compared both in living and fixed condition.
2. The fibrillar structure of the tails appears distinctly when non-agglutinated spermatozoa are observed in water. The tails of the spermatozoa of Esox consist of 11 protofibrils and those of Coregonus of 9 protofibrils. The heads, the tails and the protofibrils of the spermatozoa were measured.
3. The tail of the Esox spermium is provided with a membrane which is supposed to be contractile.
4. Upon agglutination the same phenomena are exhibited by the spermatozoa of all the investigated species: the heads stick together, the tails which are to some extent dissolved form a dense network within a slimy matrix and no protofibrils can be seen within these tails. The similarity between the agglutination of spermatozoa and bacteria is stressed.

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Die mit * bezeichneten Publikationen enthalten elektronenoptische Aufnahmen.

THE EFFECT OF STORAGE ON THE NUCLEIC ACID CONTENT AND VIRULENCE OF MOUSE ASCITES TUMOR

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Previous investigations have demonstrated that the cellular ascitic fluid obtained from mice inoculated intraperitoneally with Ehrlich carcinoma retains the ability to transmit the tumor even after prolonged storage at +4° C (4, 5, 10). In view of the observations by Caspersson and co-workers (1) on the role of ribonucleic acid in the elaboration of cell protein, it seemed of interest to investigate the changes in nucleic acid content which accompanied prolonged storage of ascites tumor cells and to attempt to correlate such changes with alterations in virulence.

METHODS

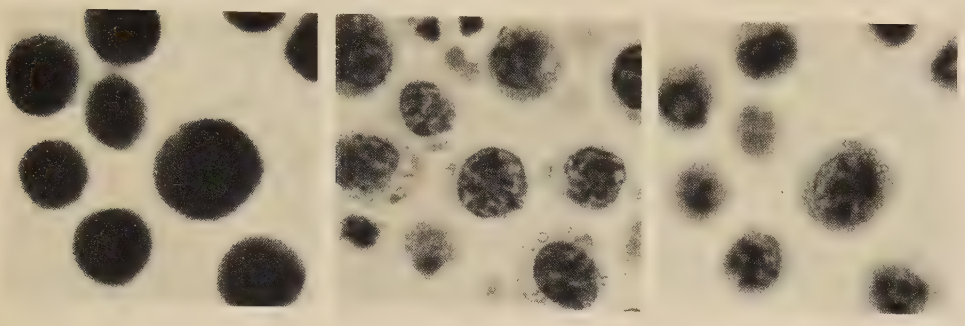
White mice of 20–25 gms were used. The original ascites tumor was obtained by inoculating intraperitoneally suspensions of cells from a solid Ehrlich carcinoma provided through the courtesy of Dr. Albert Fischer (The Biological Institute of the Carlsberg Foundation). The tumor was then maintained by intraperitoneal transfer of the ascites obtained in this manner. All animals inoculated with an adequate number of cells (over 400.000) developed ascites. If fewer cells were inoculated, the results were less uniform, and occasionally only solid tumors of the abdominal wall developed. The ascites varied in character from chylous to grossly bloody and, in the absence of anticoagulants, clotted readily. The fluid apparently has a high globulin content as indicated by the rapid production of a clot on the addition of water but not of physiological saline (6).

[1] This work was done in part (N. B. Kurnick) under a fellowship of the American Cancer Society recommended by the National Research Council, Committee on Growth.

A number of mice were inoculated with 0.2 cc (corresponding to about 20 million cells) fresh ascitic fluid. These mice regularly developed large ascites in 4–6 days and died within 10 days with 50 per cent mortality in 6 days. For each experiment, one mouse was sacrificed after 6–8 days, and the ascitic fluid (5–8 cc from each mouse) mixed with potassium oxalate (one drop of 20 per cent solution per 5 cc fluid). A small portion of this fluid was diluted with Tyrode's solution, the cells counted in a Neubauer haemocytometer; and known numbers of cells, varying from 100,000 to 21 million, were inoculated intraperitoneally into white mice, in volumes of 0.1 to 0.5 cc to determine the virulence of the fresh fluid. A 1 cc aliquot of the fresh fluid was promptly analyzed in duplicate for nucleic acid content by the method of Schmidt and Thannhauser (12). In several instances, until it became apparent that the cell-free fluid contained no nucleic acid, separate determinations were performed on whole fluid, centrifuged sediment, and supernate. A drop of the fresh fluid was also photographed in the Köhler U.V. microscope at 257 mμ which is near the maximum absorption of nucleic acids. Since it was observed that the tumor cells rapidly diminished in absorption on exposure to U.V. light, the microscope was focused in visible light and adjusted for U.V. by a predetermined shift of the micrometer screw. In this way the exposure to U.V. light occured only during the actual photography, 5–10 seconds. The balance of the fluid was stored in the refrigerator at +4° C for 9 days. At the end of the storage period a drop of fluid was again photographed in the U.V., a portion was diluted with Tyrode's solution, counted as before, and 800,000 cells inoculated intraperitoneally into groups of 10 mice which were carefully observed, with daily weighing, for the development of ascites and the dates of death noted. An aliquot was analyzed for nucleic acid as with the fresh material. Analyses were performed on whole fluid, the total sediment from a measured volume of fluid and on the supernate separately. Drops of several fresh and stored fluids were added to chicken plasma, clotted by the addition of chick embryonic extract and photographed by micro-cinematography at a rate of 2 frames per minute.

RESULTS

In the course of 9 days' storage the tumor cells showed a marked decrease in cytoplasmic absorption at 257 mμ. (Figs. 1, 2, 3.) Concomitantly there was a considerable reduction in nucleic acid phosphorus. This loss in



1. 2. 3.

Fig. 1. Fresh ascites tumor cells photographed at 257 mμ.
Fig. 2. Ascites tumor cells photographed at 257 mμ after storage at +4° C, photographed and printed under identical conditions with Fig. 1. for comparison of absorption. Note marked loss of cytoplasmic absorption as compared to fresh cells.
Fig. 3. Same as Fig. 1. printed with $^1/_3$ exposure time to show cellular details. Note that vacuoles are present in fresh cells as well as stored cells.

TABLE I

Expt	10^{-3}mgP/cc			10^6 cell/cc	10^{-9}mgP/cell			50 % mort. days	% loss	
	Total NA	RNA	DNA		Total NA	RNA	DNA		Total NA	RNA
					Fresh fluid					
1	237	158	79	39	6.1	4.0	2.1	15		
2	445	248	197	97	4.6	2.6	2.0	15		
3	525	340	185	100	5.2	3.4	1.8	13		
4	478	325	153	85	5.5	3.7	1.8	14		
5	520	294	226	95	5.4	3.1	2.3	12		
6	680	468	212	104	6.5	4.5	2.0	16		
					Stored fluid					
1	136	70	66	39	3.5	1.8	1.7	33	43	55
2	300	97	203	100	3.0	1.0	2.1	34	33	50
3	300	130	170	100	3.0	1.3	1.7	39	43	62
4	187	50	137	82	2.2	0.6	1.6	>40	61	84
5	350	150	200	96	3.7	1.6	2.1	35	33	48
6	405	177	228	103	3.9	1.7	2.2	31	40	62

nucleic acid (33 to 61 per cent) was almost entirely attributable to ribose-nucleic acid (48 to 84 per cent of the original RNA) (Table 1). The original RNA content was 2.6–4.5 $\times 10^{-8}$ mg per cell and the post-storage RNA was

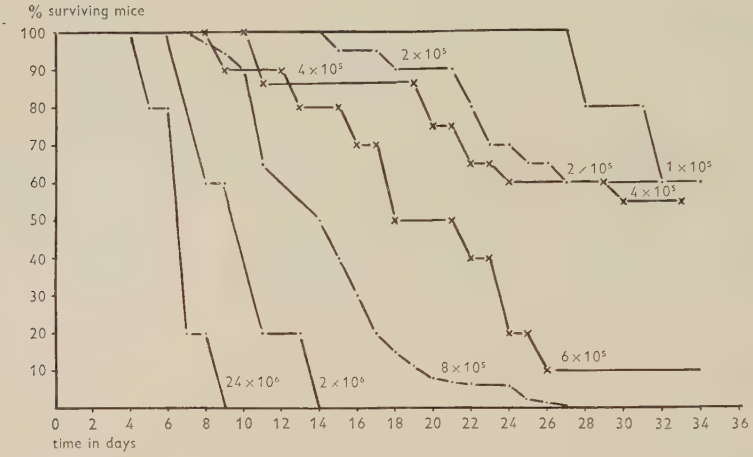


Fig. 4. Effect of number of cells inoculated on survival time of white mice. Note that earliest deaths occur later and curves become flatter as the cell number is reduced.

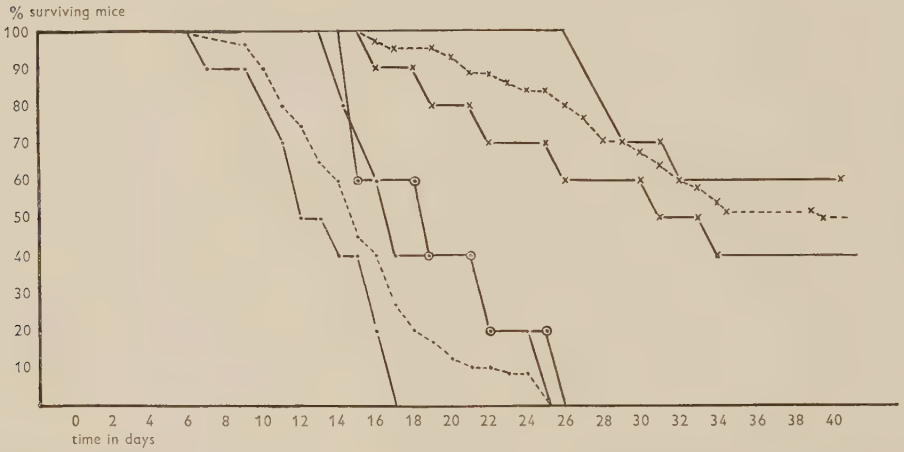
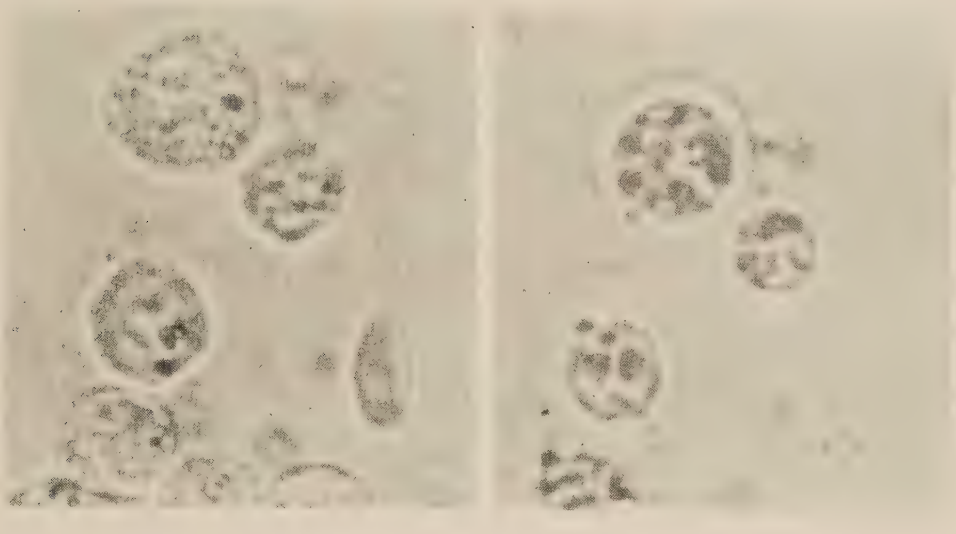


Fig. 5. Per cent survival following · —— · inoculation of 800,000 fresh cells; × —— × inoculation of 800,000 cells stored 9 days at + 4° C; ⊙ —— ⊙ inoculation of 10^7 cells stored 9 days at + 4° C (one experiment). In each case the curves plotted represent the limits observed in 6 experiments (6 donor mice) and the mean of all 6 experiments (interrupted line).

$0.6–1.8 \times 10^{-8}$ mg. The cells apparently lost their ribosenucleic acid by degradation of the nucleic acid molecule since no nucleic acid phosphorus could be found in the supernate and all of the nucleic acid of the fluid was present in the cell sediment.



6. 7.

Fig. 6. Print from cinematogram of surviving ascites cells in clotted chicken plasma.
Fig. 7. Same cells after storage at 37° C overnight.

A curve of the death rate (Fig. 4) following the inoculation of fresh ascitic fluid reveals that animals inoculated with 21 million cells or more begin to die on the 5th day, reach a mortality of 50 per cent on the 6th day, and are all dead on the 9th day. With 2 million cells the first deaths occur on the 7th day, 50 per cent mortality occurs on the 9th day and 100 per cent on the 14th. Eight hundred thousand cells is followed by a 50 per cent mortality on the 14th day. Reducing the inoculum to 400,000 cells results in a prolongation of the 50 per cent survival to the 30th day. The inoculation of 800,000 cells which had been stored 9 days (Fig. 5) produced a 50 per cent mortality only after 30 days or longer, and to obtain 50 per cent mortality on the 18th day required the inoculation of 10 million cells. Thus storage is followed by a reduction in virulence characterized by a prolongation in survival. Furthermore, unlike the results with fresh cells, the inoculation of 800,000 stored cells occasionally produced solid tumors without ascites (compare effect of inoculation of less than 400,000 fresh cells). This was most marked in the experiment in which storage resulted in the greatest reduction in RNA (84 per cent), where only 30 per cent of the animals had developed ascites in 40 days. In the other experiments with 48–62 per cent reduction in RNA, 50 per cent of the animals died between 31 and 39 days.

This suggests that the percentage loss of RNA may be directly correlated to the loss of virulence.

Cinematography revealed motility in fresh and stored cells. Cells which were allowed to die by standing at 37° C overnight showed no motility, a clear peri-nuclear space, and denser, more heterogenous nuclei (Figs. 6, 7).

DISCUSSION

In an attempt, such as this, to correlate changes in nucleic acid content and virulence, it is important first to consider whether or not the cells survive the storage period. The ultra-violet photographs demonstrate a uniform loss of absorption in all cells, so that the possibility of the loss of nucleic acid by only a portion of the cells which may have died without the participation of other surviving cells is excluded. The problem, then, is to decide between 1. the death of almost all the cells with subsequent nucleic acid loss and 2. the loss of ribosenucleic acid by surviving cells. A number of arguments favor the second possibility. Firstly, there is the fact that the stored fluid is capable of inducing the tumor at all. Like other investigators (10, 7) we have been uniformly unsuccessful in attempts to induce the tumor with fluids which are known not to contain considerable numbers of living cells. The supernates from centrifuged ascites fluids were avirulent. Frozen-dried ascites cells also failed to induce the tumor. Furthermore we disrupted the cells of a fresh ascites fluid mechanically in a Waring Blendor (during which process the temperature was not permitted to exceed +15° C). Blending was continued until very few or no intact cells could be found in the fluid (about 9 minutes). None of several large series of animals inoculated with a volume of this fluid containing the remnants of about 3 million cells developed ascites in a 3 month observation period, while all of the animals inoculated with 800,000 unbroken cells died of ascites within 20 days (8). Secondly, whereas dead cells could be expected to autolyze, the cell number after storage was not found to have altered. Finally, the preservation of the DNA in the face of RNA depletion points to the survival of the cells. One of us (9) has observed the same phenomenon in the liver cells of rats and rabbits on prolonged starvation. Here too, there was a depletion of RNA (and protein) both in nuclei and cytoplasm, while the DNA remained unchanged.[1] It would appear that a constant nuclear DNA content is character-

[1] This agrees well with the results of previous investigators, summarized by Davidson (3).

istic of living cells. In the case of mice, rabbits, and rats (9) the DNA content of liver and kidney nuclei was found to lie between $0.7–0.9 \times 10^{-8}$ mg DNA per cell. Mirsky and Ris (11) and Vendrely and Vendrely (13) have published similar figures for other mammalian somatic cells. It is of interest that the tumor cells have a much higher DNA content (2.0×10^{-8} mg DNA per cell), perhaps indicating a high degree of polyploidy. As in other somatic cells the DNA content of ascites cells was the same in all the animals studied despite great physiological and experimental variations in RNA.

It appears, then, that the tumor cells, during prolonged storage in the cold, survive at the expense of their own RNA and probably also protein. This state of depletion results in a diminution in virulence with a shift in the mortality curve of inoculated mice, toward longer survival. Caspersson and Santesson (2) have studied a number of solid mammalian carcinomas by U.V. microscopy and have concluded that the tumor cells fall, generally, into two classes: 1. the A cells lying near the nutrient vessels, rich in cytoplasmic RNA, and showing rapid growth and 2. the B cells, at a distance from the nutrient vessels, poor in cytoplasmic RNA, and sluggish in growth. Our observations are consistent with this correlation of RNA content and rapidity of growth. The fresh tumor cells rich in RNA like the A cells, show rapid production of ascites tumor and early lethal effect; the stored tumor cells, having become depleted in a poor nutrient environment, like the B cells, reveal slower tumor production with prolonged survival of the host. It is of interest that variations in RNA content between 2.6 and 4.5×10^{-8} mg RNA per cell observed in fresh ascites fluids are not associated with significant variations in virulence. However, reduction of RNA below 1.8×10^{-8} mg RNA (the highest observed in stored cells) is associated with considerable loss in virulence.

SUMMARY

The effect of storage for 9 days in the cold on the nucleic acid content and virulence of ascites tumor cells was studied by chemical and U.V. methods. It was found that storage was accompanied by considerable loss of cellular RNA but not of DNA. Correlated with the loss of RNA is a reduction in virulence. Evidence is presented to indicate that the cells survive the storage period. The high constant DNA content as compared to other somatic cells may possibly be interpreted as evidence of polyploidy in ascites tumor cells.

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BRIEF NOTES

8-ETHOXYCAFFEINE, AN IDEAL INDUCER OF STRUCTURAL CHROMOSOME CHANGES IN THE ROOT TIPS OF ALLIUM CEPA

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In a previous publication (Kihlman 1949) there is a description of the effect of a number of purine derivatives on *Allium* chromosomes, expressed in per cent of abnormal anaphases (% AA). When the investigation was continued, it appeared that a certain group of purines differs from the other as to the mode of action. This group consists of three of the four 8-ethers of caffeine which have been synthesized, viz., 8-ethoxycaffeine, 8-allyloxycaffeine, and 8-propyloxycaffeine. The fourth, 8-methoxycaffeine, agrees more, as to its effect, with the other active purine derivatives, i.e., it acts in such a way as to obtain the highest % AA in a comparatively low concentration, which permits some mitotic activity even during a long treatment. Ethoxycaffeine, allyloxycaffeine, and propyloxycaffeine act rapidly, and the effect, which on the whole seems to increase in proportion to their concentration, is independent of mitotic activity.

The action of these 8-ethers of caffeine may be explained by the hypothesis that, in contrast to other active purine derivatives, they are able to penetrate the membrane of the cell nucleus, and thus to act on interphase chromosomes. If a purine derivative is to have a strong effect, it is necessary according to this hypothesis to fulfil the two following conditions: it must be able (1) to induce structural chromosome changes, and (2) to act on the interphase chromosomes. The former property is connected in some

way with the solubilizing power of the purine derivatives (Kihlman 1949), the latter perhaps with their solubility in lipids. More profound investigations are, however, needed before it can with any certainty be decided on what the difference in action between the three 8-ethers of caffeine and other active purine derivatives depends.

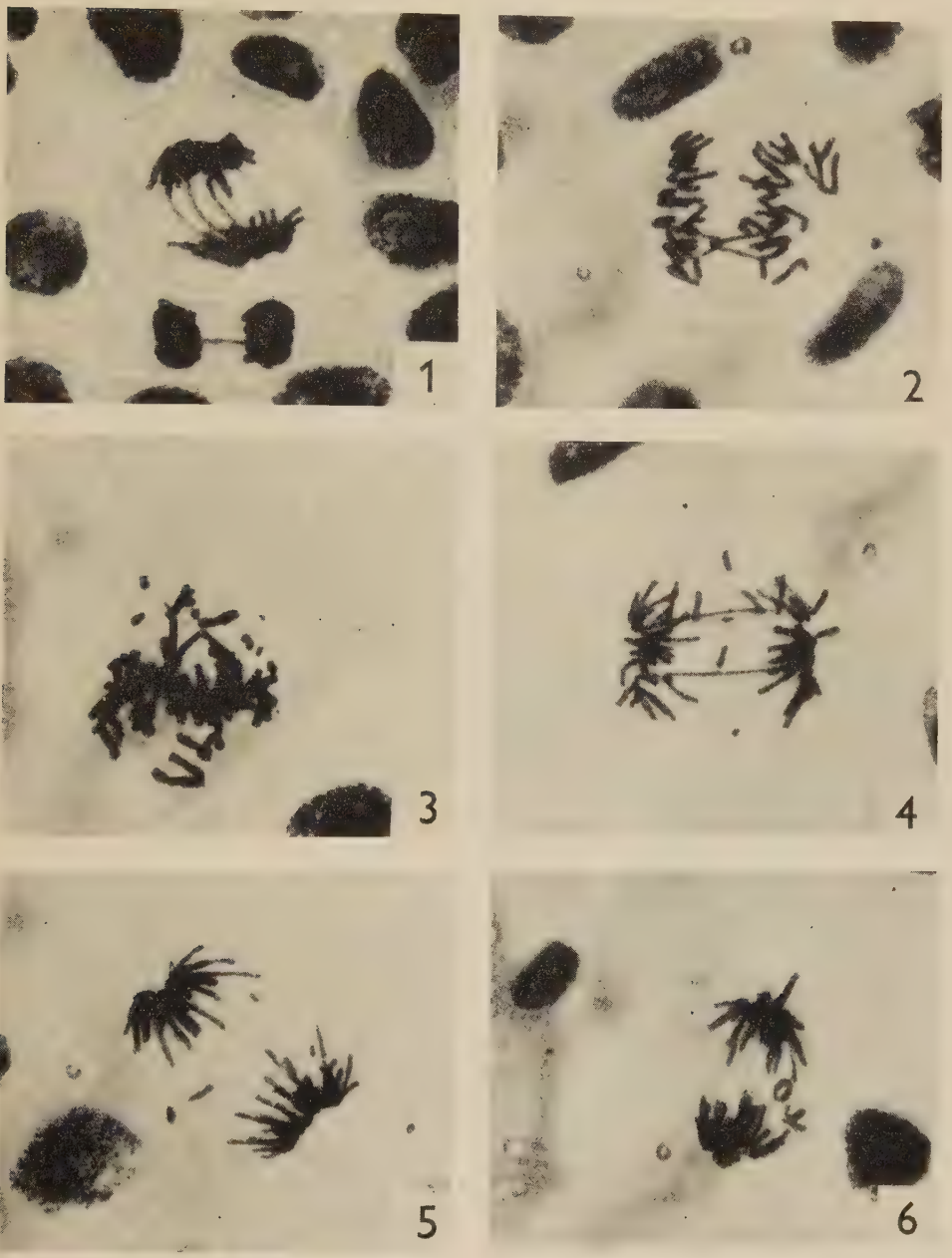
The best results were obtained with ethoxycaffeine. A comparatively high water solubility, which permits a treatment with stronger concentrations, and a low toxic effect makes it possible to obtain a very high % AA by means of this purine derivative. (Although the effect of tetramethyluric acid *per mmol of purine* is about twice that of ethoxycaffeine, the latter purine derivative may induce a % AA that is about three times greater than the highest % AA obtainable by tetramethyluric acid after 24 hours' treatment.)

The effect of ethoxycaffeine has been investigated after ½, 1, 2, 4, 8, 16, 24, and 32 hours' treatment in a 0.24 per cent (10 mmol/l) water solution. Fixations in acetic alcohol were made immediately prior and subsequent to the treatment. Then the bulbs were transferred to water, and fixations were made every 12th hr. for at least three days. The material was stained according to the Feulgen squash method, and mounted in Canada balsam.

The first time after the bulbs had been transferred into water, no mitosis occurs in the root tips. The period without mitosis lasts, as a rule, from 12 24 hrs. During this time temporary alterations, e.g. the stickiness phenomenon, have disappeared.

After all the periods of treatment mentioned here, structural chromosome changes can be observed as soon as the mitosis has begun. After a treatment of ½ hr. about 15 % AA can be obtained, and after that the effect increases rather continually, to reach its highest value of about 80 per cent after a treatment of 4–8 hrs. With longer treatments the % AA is rather constant, showing only such changes as depend on the varying susceptibility of the onions. When the treatment is prolonged, however, the effect becomes more extreme. After ½–2 hrs. of treatment only one or a few chromosome and chromatid breaks per AA may be observed, but as soon as after a treatment of 4 hrs. there are usually considerably more breaks and the chromosomes are frequently fragmented to such an extent that anaphase movement is no longer possible. In general, the % AA remains rather unaltered during at least 24 hrs. after the first appearance of the effect.

The ability of ethoxycaffeine to induce structural chromosome changes in lower concentrations than 0.24 per cent has so far been investigated only



Figs. 1–6. For explanation, see text. Magnification, 1,000 ×.

incompletely. After a treatment of 24 hrs. in 0.12 per cent (5 mmol/l) about 40 % AA was obtained, and after an equally long treatment in a 0.05 per cent (2 mmol/l) solution the highest value obtained is 6.8 % AA. As expected, the effect in lower concentrations is not very extreme, and usually only a few breaks per cell are obtained.

Most of the structural chromosome changes known from X-ray experiments are observed after a treatment with ethoxycaffeine. Some microphotographs were taken to illustrate the effect. Fig. 1 shows physiological changes (sticky bridges in telophase) obtained after a treatment of 4 hrs. with a 0.01 per cent (0.5 mmol/l) solution, and Figs. 2–6 show structural chromosome changes obtained after 2–24 hours' treatment with a 0.24 per cent solution.

Since, in a nearly ideal way, ethoxycaffeine combines a strong effect on chromosomes with a high permeability and a low toxicity, it seems to be an excellent inducer of structural chromosome changes. Further, the treatment is easily performed, and the synthesis of ethoxycaffeine from caffeine causes no difficulties.

Investigations of the eventual mutagenic effect of ethoxycaffeine are carried out at this Institute.

I am greatly indebted to dr Lars Eric Henriksson for taking the microphotographs.

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THE DISTRIBUTION OF FOOD IN AMOEBA CYTOPLASM STUDIED BY MEANS OF AUTORADIOGRAPHY[1]

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THE manner in which the contents of food vacuoles are incorporated in the cytoplasm of amoebae has been subject to divergent interpretations. Mast and his school (4, 5, 6) maintain that the food is transformed *within the food vacuoles* into various cytoplasmic inclusion bodies (mainly crystals, fat and refractive bodies) which subsequently, by division of the vacuoles, are spread throughout the interior of the amoeba. Andresen and Holter (1), on the other hand, suggested that the products of digestion are taken up primarily by the hyaline cytoplasm and that inclusion bodies are formed in the cytoplasm and not in the food vacuoles.

In the hope of elucidating this problem we have studied the distribution of food material labeled with carbon-14. The radioactive carbon was incorporated by assimilation in algae (*Chlorella* sp.) which live as symbionts in the cytoplasm of a large ciliate, *Stentor polymorphus*. When the activity of the stentors had reached an average value of

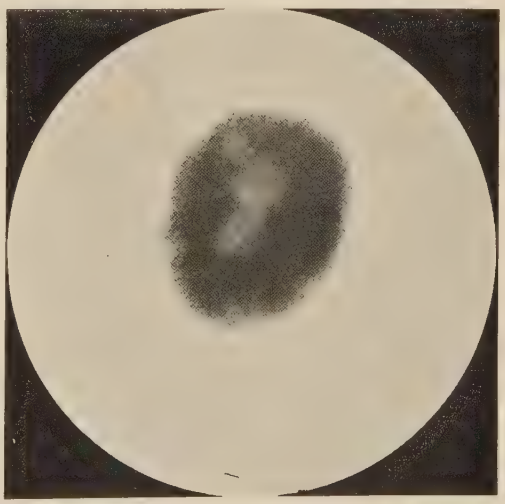
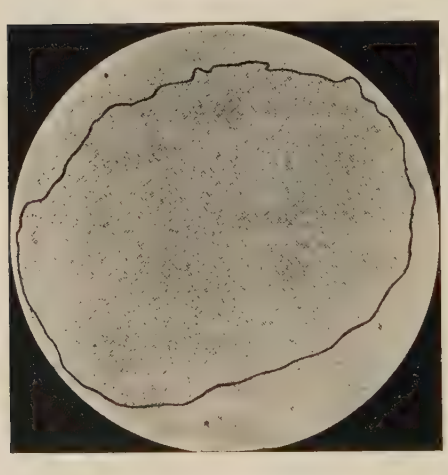


Fig. 1. Food vacuole containing one radioactive stentor immediately after ingestion. 10 μ section, exposure 20 days, magnification 200 $\times$. Activity of whole stentor ca 200 c/m. Note that the algae (the centers of activity) are situated at the periphery of the stentor.

[1] Supported by grants from the Danish Anti-Cancer League and P. Carl Petersen's Foundation.

Fig. 2. Food vacuoles 2 hrs 30′ after ingestion of 2 stentors. 10 μ section, exposure 26 days, magnification 200 ×. Total activity of stentors ca 900 c/m. The contents of the vacuoles have been compressed and the vacuoles are beginning to divide.



283 counts per minute per individual, as determined with an end window counter (diameter 2.7 cm, window c. 3 mg/cm^2) at a distance of c. 3 mm from the window, the stentors were fed to amoebae (*Chaos chaos* (2)). At various intervals after ingestion, the amoebae were fixed with or without previous centrifugation (3,000 ×g), cut, and the slices brought into intimate contact with Eastman NTB film. The technique was similar to that described by A. M. MacDonald, J. Cobb, and A. K. Solomon (3). The exposure time was 14–26 days.

Fig. 3. Cytoplasm of amoeba 60 hrs after ingestion of 2 stentors. 10 μ section, exposure 26 days, magnification 200 ×. Total activity of stentors ca 250 c/m. The food vacuoles have disappeared and the radioactivity is evenly distributed. The outline of the amoeba has been drawn from the underlying section (focus difference 10 μ).

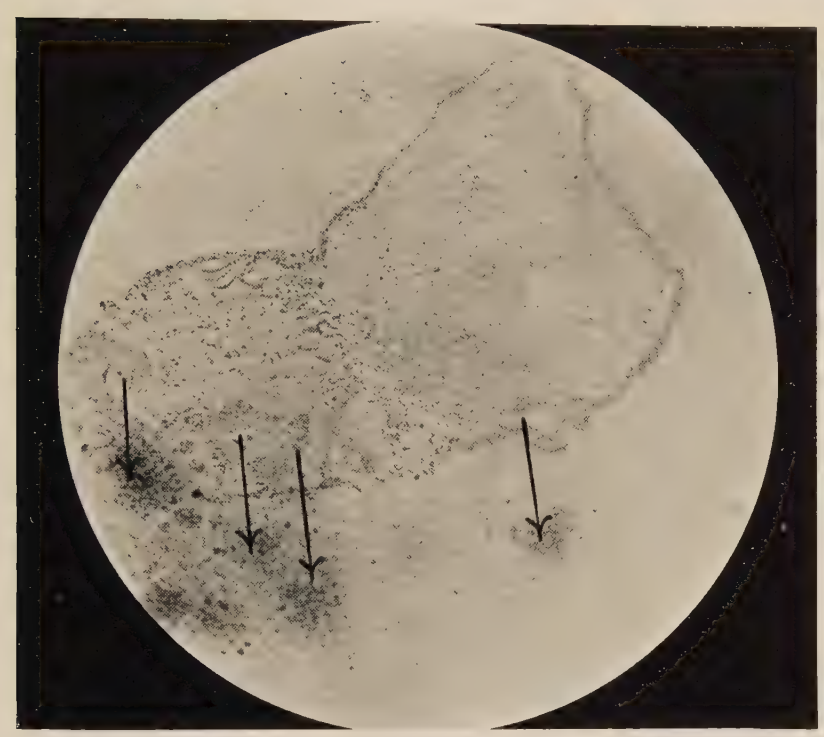


Fig. 4. Amoeba centrifuged 3 hrs after ingestion of 3 stentors. 10 μ section, exposure 14 days, magnification 200 ×. Total activity (measured on sections) 466 c/m. The picture was chosen on account of a slight lateral dislocation of the film during development so that both section and autoradiogram can be shown on one picture.

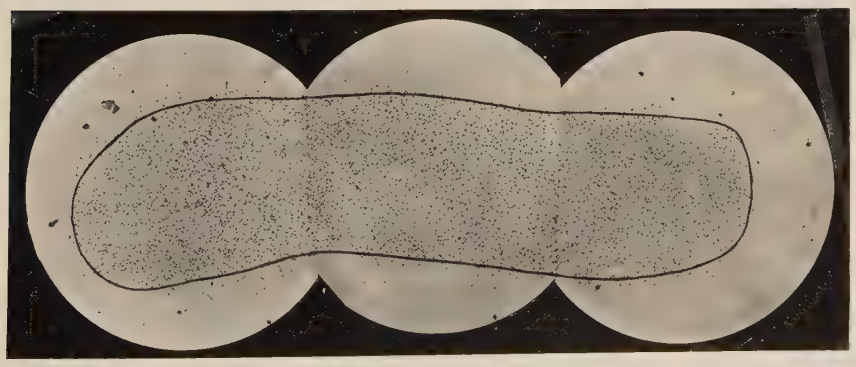


Fig. 5. Amoeba centrifuged 120 hrs after ingestion of 2 stentors. 10 μ section, exposure 26 days, magnification 200 ×. Total activity of stentors ca 900 c/m. Mounted from 3 photographs. Centrifugal pole to the left. Note even distribution of activity; only th outline of the amoeba section, but not the stratification could be drawn.

From Figs. 1–3 (uncentrifuged amoebae) it follows that during early stages of digestion the radioactive material is confined to the food vacuoles, but after 60 hours it is uniformly distributed over the whole amoeba. This latter result may indicate that the active material has been taken up by the hyaloplasm; however, the same picture would have been obtained if the activity were localized in those inclusion bodies (mitochondria, crystals, etc.) which are distributed more or less uniformly over the cytoplasm. The resolution of the autoradiographs does not permit the separation of foci less than about 20 μ apart.

In centrifuged amoebae, where the cytoplasmic inclusions mentioned are collected in layers according to their specific gravity, the situation is different. From Figs. 4 and 5, however, it can be seen that the picture is still essentially the same. As long as the food material is mainly confined in vacuoles, radioactivity is accumulated in the heavy stratum where the food vacuoles are collected. In later stages (corresponding to Fig. 3) the activity remains uniformly distributed even after centrifugation. This means that the active material is localized either in the hyaloplasm or in some kind of inclusion bodies that are not stratified by the centrifugal forces employed.

The evidence of these preliminary experiments thus seems to support the assumption that the crystals etc. are not formed within the food vacuoles. As suggested previously (1), it is more likely that the food material is transferred primarily to the cytoplasm to join the general metabolism of the amoeba.

The experiments reported so far are purely qualitative. It is our impression that a more quantitative study of these problems by means of autoradiography is not entirely impossible. The experiments are continued and will later be reported in detail.

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ELECTRON MICROSCOPE OBSERVATIONS ON THE STRUCTURE OF THE MYELINATED NERVE FIBER SHEATH[1]

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The results of polarization optical analysis (Göthlin (7); Schmidt (10); Chinn and Schmitt (2)) and of x-ray diffraction studies on nerves (Handovsky (8); Boehm (1); Schmitt (11); Schmitt and Bear (12)) have led to a conception of the myelin sheath's submicroscopic structure which is sketched by Schmitt, Bear and Palmer (13): "The sheath is described as being composed of concentrically wrapped layers of mixed lipides alternating with thin, possibly unimolecular, layers of neurokeratinogenic protein material." Attempts have been made to observe the postulated foliate structure directly with the electron microscope, working on nerves subjected to various fragmentation procedures (DeRobertis, Sjöstrand (4)). Both these investigators and the author obtained pictures of fragmented lamellae piles which could be traced to the myelin sheath based only on indirect evidence. It has now been possible to demonstrate these lamellar structures in situ, within the myelin sheath, and to study their relation to the fibrous components of the nerve sheath, by examining extremely thin frozen sections of fixed medullated nerve fibers. Using a modification of the freezing-expansion technique of Newman, Borysko and Swerdlow (9), transverse, longitudinal and tangential sections of nerve fibers, thin enough for electron microscopy (0.3–0.1 μ) can be cut. Embedding artefacts are thus largely avoided and whole, well preserved sections of nerve made available for examination with a minimum of preliminary treatments. Under these conditions the submicroscopic elements previously revealed with fragmentation methods can generally be studied directly in their original position.

Material and Methods: The sciatic nerve of the albino rat was mainly used, in addition to peripheral and cranial nerves of the bullfrog, human motor roots and the olfactorius of the pike. These nerves were examined after fixation in osmic acid (1 per cent, 2 per cent), Formalin (10 per cent) and in the fresh state. Suitably oriented whole

[1] Extract of a paper read at the Department of Cell Research on Nov. 15th, 1949.

nerve segments are mounted on the stage of the freezing expansion chamber attached to a Spencer Rotary microtome, and sections of 1—0.1 micron cut with special razor blades after deep freezing of the knife holder. Sections are picked up directly on the el. microscope screens covered with collodion or beryllium films; they are also spread out on water. After washing carefully the sections can be stained with phosphotungstic acid or shadowed lightly with Platinum-Palladium at an angle of 5 —20°. Frozen sections of fresh, unfixed nerve adhere very firmly to glass slides and can be subjected to a modification of the Replica-Adhesion technique (6, 5) after fixation with osmium vapors. In this way coherent sections thinner than 0.1 micron were prepared. Thin sections of nerves embedded in celloidin-paraffin are used for control purposes. Finally, Replica-Adhesion sections of microdissected single nerve fibers and digestion experiments with enzymes are among the numerous control procedures employed in the course of systematic studies to be published later. All observations were made with an RCA EMU model electron microscope.

RESULTS

1) *The Myelin Lamellae.* The bulk of the myelin sheath breaks down into piles of thin, transparent lamellae of uniform appearance, their shape depending upon the plane of section. Thus in longitudinal sections (Fig. 1) long, laminated slabs of thin plates, with jagged, staircase-like ends, extend into the lumen of the fiber; while in transverse sections thin ribbons of lamellae peel off from the dense rings which represent the myelin sheath. The continuity of the thin lamellae with the dense foliated mass of the sheath can be followed clearly in numerous transitions. When isolated in large numbers by gentle fragmentation of relatively thick (1–5 μ) frozen sections (Fig. 2) the thinnest or unit lamellae are 100–150 Å thick and several microns long; up to 10 of them can be distinguished piled closely upon each other. They are best preserved in Osmium fixed preparations where they appear like transparent, flexible films with a tendency to crease and double up, but with no definite indications of surface structure. After light metal shadow casting, however, a characteristic surface structure is seen, consisting of 50–100 Å granules arranged in a regular pattern. Occasionally, droplets (200 Å diam.) resembling knobs are found on the free surfaces in both Formalin and Osmium fixed material. These surface structures may correspond to the radially oriented lipides, but the possibility of fixation artefacts cannot be ruled out yet. Fenestrated or finely perforated, porous lamellae are not uncommon even in well fixed preparations, and their granular or droplet-like disintegration products are similar to the surface structures just described. Measurements carried out on shadowed specimens indicate that the lamellae themselves, devoid of the granular surface structure, are

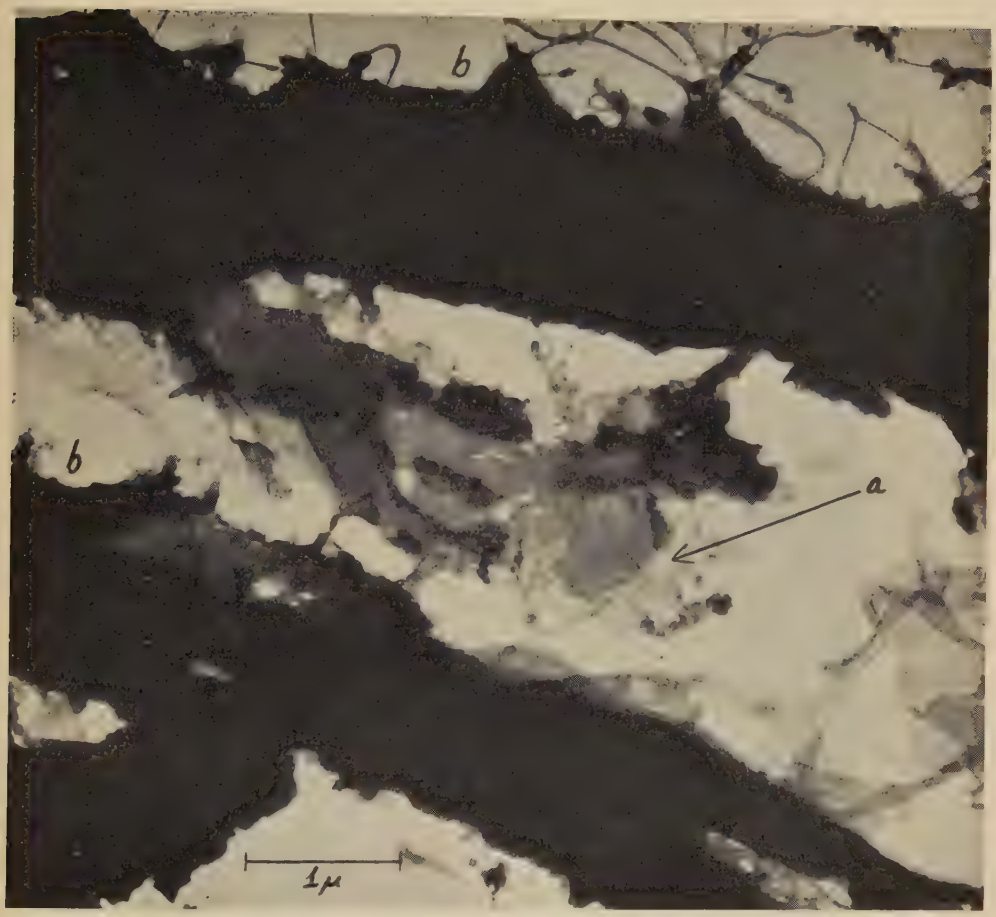

Fig. 1. Longitudinal section through a single nerve fiber of the rat sciatic, showing long bands of myelin lamellae (a) extending from the two dense myelin sheath columns (b) into the lumen. Osmic acid fix. Electron micrograph. Magnification: 20,000 ×.

not more than 50 Å thick and protein-like in appearance, while the latter contribute the remaining 50–100 Å. The thicker piles are built up of unit plates with an average thickness of 100–150 Å, the variations depending upon the presence of a diffuse substance spread out between the individual lamellae and forming irregular patches reminescent of liquid films trapped between glass panes. From the evidence at hand it is difficult to say how these lamellae are arranged within the sheath. They are generally found stacked in solid piles or curved slabs not exceeding several microns in length,

Fig. 2. Myelin lamellae isolated from a longitudinal section. Rat sciatic. Notice numerous folds in the lamellae and indications of surface structure. Osmium fix. Platinum/palladium shadowed. Electron micrograph. Magnification: 24,000 ×.

embedded between the outer and inner fibrous layers and arranged more or less concentrically. A discontinuous arrangement prevails in longitudinal sections, giving rise to a segmented "wave" pattern of myelin plate inclusions which are separated by fibrous compartments. (But this may well be the result of cracks and distension produced in the fragile myelin component by sectioning.) In shadowed replicas of isolated single nerve fibers the external

layers of myelin exhibit a scaly surface structure, formed by densely packed small flakes. The identity of the described lamellar structures with the concentrically wrapped submicroscopic lipide-protein layers postulated in the myelin sheath to account for the results of polarization optical analysis and of x-ray diffraction studies, seems highly probable, even as regards the quantitative data (fundamental x-ray diffraction spacing of 158 Å for dried mammalian medullated nerve compared with the 100–150 Å thick unit myelin lamellae). The myelin lamellae have also certain features in common with the unit discs of the outer segments of guinea pig retinal rods described by Sjöstrand (14).

2) *The fibrous layers of the nerve sheath.* Transverse sections through nerve fibers reveal two distinct fibrous layers adhering closely to the central, lamellar portion of the sheath: (a) an external granular membrane covered with typical collagen fibrils and corresponding mainly to the Neurilemma and the adjacent connective tissue; (b) an internal layer of densely woven thin fibrils connecting the myelin sheath with the fibrous structures of the axon. This layer can probably be correlated with Mauthner's sheath. Both layers are studied best in longitudinal or tangential sections. (a) *The external layer* separates easily from the myelin proper and consists of a compact finely granular membrane (approx. 200 Å thick) covered with collagen fibrils running single or in bundles longitudinally and in the transverse direction, or interlaced obliquely according to the stretched condition of the fiber. (Fig. 3.) The collagen fibrils of this layer are frequently tubular in appearance, *resembling the "neurotubuli"* described by DeRobertis and Schmitt (3), *but only in Formalin fixed material and when they are embedded in a hazy (lipide?) substance* flowing from the myelin sheath and contaminating the background. The abundance of these collagen fibrils adhering so closely to the nerve sheath and arranged in longitudinal bundles can easily be mistaken for fibrillar components of the axon, specially in replica-adhesion preparations. In none of the specimens examined could structures resembling the neurotubuli be found within the axon. Instead, the axon contained a very fine network fibers 100 Å thick stretched out in a transverse or oblique transverse direction. *A special type of extremely thin (100 Å diam.) smooth fibrils,* with no detectable periodicity, bound together in compact strands, are regularly seen attached to the collagen fibrils of the outer layer. (b) *The internal layer* consists of a dense framework of thin (100–200 Å) fibrils with broad, irregular reinforcements. This layer is attached firmly to the myelin component and extends into the axon, where it forms the basis for the fine cobweb-like network bridging the lumen of the

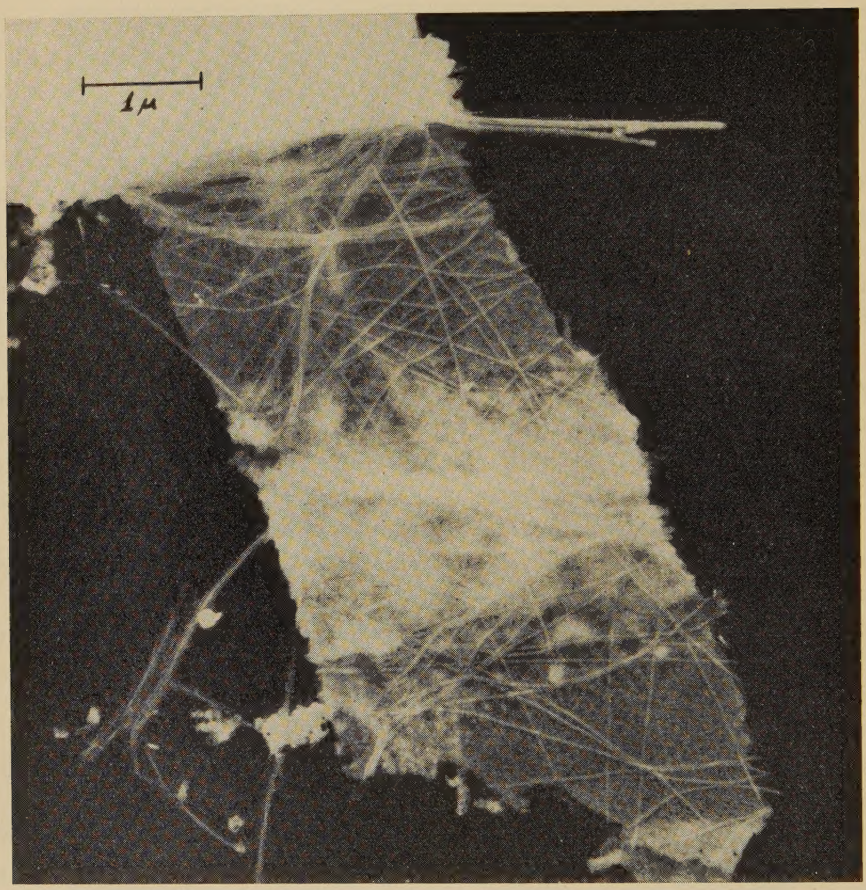

Fig. 3. External sheath membrane (Neurilemma) with interlaced collagen fibrils lying on top. Tangential section, rat sciatic. Osmic acid fix. Platinum/palladium shadowed. Electron micrograph. Magnification: 15,000 ×.

axis cylinder. The structures described are located in the internode portion of the fiber, since the nodes have not been adequately prepared yet. Although numerous artefact possibilities (dislocation and distortion of structures when cutting and mounting the sections, fixation artefacts, etc.) must be considered, the preliminary findings presented here were consistently reproducible with different methods. The investigations are being continued with different nerve types and a complete description of the techniques and results will soon be published.

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